Studies on the mechanism of spermatocyte-specific gene activation by enhancer and long noncoding RNA during mouse spermatogenesis

（マウス精子形成におけるエンハンサーとlong noncoding RNA による精母細胞特異的遺伝子活性化メカニズムに関する研究）

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CONTENTS

GENERAL INTRODUCTION

The mammalian testis is composed of three types of cells, germ cells, Sertoli cells, and Leydig cells, and they directly or indirectly interact with each other for normal progression of spermatogenesis after birth. Sertoli cells play critical roles in many aspects of spermatogenesis, such as germ cell migration into the lumen (Bergmann et al. 1989), construction of blood testis barrier (BTB) (Dym & Fawcett 1970, Pelletier 2011), and maintenance of germ line stem cells (Li et al. 2009). Leydig cells are responsible for steroidogenesis, and the secreted androgen regulates the BTB structure, Sertoli-germ cell adhesion, and the release of sperm (O'Shaughnessy 2014). Androgen production and secretion from Leydig cells are controlled by pituitary gland in response to gonadotropin releasing hormone (GnRH) from the hypothalamus (Zirkin & Chen 2000). This regulation by hypothalamus-pituitary-gonadal axis is very important, as follicle stimulating hormone (FSH) from the pituitary and testosterone from Leydig cells are both known to prevent apoptosis of germ cells (Erkkilä et al. 1997, Tesarik et al. 2001). In addition to the endocrine control, testis-specific genes play essential roles in the regulation of spermatogenesis, as evidenced by several studies showing the requirement of them (Table 1).

Spermatogenesis consists of three steps; mitosis of spermatogonial stem cells with a chromosome replication and a division into two daughter cells, meiosis with a DNA replication generating four haploid spermatids from primary spermatocytes by two divisions, and spermiogenesis by which round spermatids transform into mature spermatozoa through the step of spermatid elongation along with transition of histones into protamines. All of these issues are important and have been studied for many years. For example, a serine-threonine kinase, mammalian target of rapamycin (mTOR), promotes the self-renewal of spermatogonia (Xu et al. 2016), and some growth factors and transcription factors from Sertoli cells, such as glial cell line-derived neurotrophic factor (GDNF), are known to function upstream of mTOR (Chen & Liu 2015). The transition of histones into protamines has also been a critical issue, and chromatin remodeling proteins, histone modification enzymes, and

testis-specific histone variants are found to play critical roles (Table 1). However, there are still many unsolved issues in the generation of functional sperm, and one such topic is the activation mechanism of meiosis-specific genes. Thus, I attempted to solve this problem in my PhD course study.

The activation mechanism of male germ cell-specific genes in meiosis, is so important in spermatogenesis, because many of such genes have been demonstrated to be critical for the proceeding of each meiotic stage (Table 1). But the gene activation mechanism, especially during meiosis, is not well understood. In general, the tissue-specific gene activation involves various factors, such as promoters, enhancers and silencers, epigenetic modifications, chromatin remodeling, and noncoding RNAs (ncRNAs). Although some germ cell-specific genes were considered to be controlled solely by their proximal promoters (Kehoe et al. 2008, Reddi et al. 1999), recent studies have shown that other factors such as enhancers are required for several germ cell-specific genes (Kurihara et al. 2014, Lele & Wolgemuth 2004). To gain more insights into the activation mechanism of stage-specific genes in meiosis, I focused on the gene which is specifically activated in primary spermatocytes, because this stage is the longest period in spermatogenesis and numerous important genes are activated in primary spermatocytes. Here, I used the *Protease serine/Testis-specific serine protease* (*Prss/Tessp*) gene cluster that are activated at early stages of meiosis as a model gene locus.

Prss/Tessp genes encode serine proteases that are specifically expressed in the mouse testis. Four homologous genes were found from 8-week-old mouse testes by reverse transcription-polymerase chain reaction (RT-PCR) with degenerate primers designed at conserved sequences among serine proteases and by screening a mouse testis cDNA library. They were named *Prss41/Tessp-1*, *Prss42/Tessp-2*, *Prss43/Tessp-3*, and *Prss44/Tessp-4*. While *Prss41/Tessp-1* is located on the chromosome 17 of mouse as one gene in a serine protease gene cluster (Wong et al. 2004), the other three *Prss/Tessp* genes are composed of another gene cluster on chromosome 9F2-F3. *Prss41/Tessp-1* is expressed in spermatogonia and primary spermatocytes as well as Sertoli cells, and it might

contribute to the progression of spermatogenesis in spermatogonia (Takano et al. 2005, 2009; Yoneda & Kimura 2013). Transcripts of the three *Prss/Tessp* cluster genes are expressed mainly in spermatocytes, but their protein localization was different from each other (Yoneda et al. 2013). The Prss42/Tessp-2 protein is present in the membrane and cytoplasm of secondary spermatocytes and spermatids, whereas the Prss43/Tessp-3 protein is found in the membrane of primary and secondary spermatocytes. The Prss44/Tessp-4 protein is localized in the cytoplasm of spermatocyte. *Prss42/Tessp-2* and *Prss43/Tessp-3* were functionally analyzed by adding the specific antibody to *in vitro* organ culture system, and the results showed that the antibody against *Prss42/Tessp-2* and *Prss43/Tessp-3* arrested *in vitro* meiosis at the stage of secondary and primary spermatocyte, respectively, leading to germ cell apoptosis (Yoneda et al. 2013). These strongly suggest that the *Prss/Tessp* genes are functionally crucial for spermatogenesis, and therefore, this gene cluster is an ideal model to investigate the activation mechanism of spermatocyte-specific genes.

In mammalian genome, approximately 25,000 protein-coding genes are transcribed into mRNAs (Bult et al. 2016), but they correspond to no more than 2% of the whole genome, and a large amount of the rest of it (~62%) is transcribed into ncRNAs (Heward & Lindsay 2014). ncRNAs are generally defined as transcripts that do not encode proteins but act as functional molecules. They have been considered as junk for a long time, but resent studies have revealed their relation to and crucial roles in various biological processes (Kung et al. 2013, Quinn & Chang 2016). ncRNAs are roughly divided into two categories by their length, i.e. small ncRNAs and long ncRNAs (lncRNAs). Small ncRNAs have a long history of research since the discovery of the first microRNAs (miRNAs) as regulators of developmental transition in *Caenorhabditis elegans* in 1993 (Lee et al. 1993, Pasquinelli & Ruvkun 2002), and have been studied over 20 years. Small ncRNAs are less than 200 nucleotides (nt) in length, and include miRNAs, transfer RNAs (tRNAs) , small nucleolar RNAs (snoRNAs), and Piwi- interacting RNAs (piRNAs) (Kung et al. 2013, Romano et al. 2017). It is now revealed that the functions of small ncRNAs are relatively limited depending on the class. For example, miRNAs

control the translational repression and mRNA turnover (Ameres & Zamore 2013), and piRNAs regulate the repression of transposable elements and mRNA decay in murine germlines (Ng et al. 2016).

In contrast to small ncRNAs, functions and regulatory mechanisms of lncRNAs are not clear and presumed to be various (Dey et al. 2014). It is defined that lncRNAs are longer than 200 nt, frequently 5'-capped and polyadenylated, and transcribed by the similar system to mRNAs of protein-coding genes. Since the cross-species conservation rate of lncRNAs is low (Wang et al. 2004) and their primary sequences are quickly evolved compared with protein-coding genes (Pang et al. 2006), it is considered that the secondary structure is critical for their activity (Johnsson et al. 2014). In addition, lncRNAs show more tissue-specific expression patterns than mRNAs and the testis contains especially large amounts of lncRNAs (Cabili et al. 2011, Necsulea et al. 2014). As their examples, lncRNAs include long intergenic RNAs (lincRNAs), enhancer RNAs (eRNAs), antisense RNAs, and promoter-associated ncRNAs (pancRNAs) (Heward & Lindsay 2014, Kung et al. 2013, Morlando et al. 2015). These lncRNAs are reported to play important roles in RNA processing (Tripathi et al. 2010), regulation of RNA stability (Yang et al. 2014), construction of subnuclear structures (Nakagawa et al. 2014, Standaert et al. 2014), and chromatin remodeling (Zhu et al. 2013a), mostly in somatic cells. However, the classification of lncRNAs is still confusing because of their structural and functional variability, so it is meaningful to reveal the function of each lncRNA to understand what the lncRNA is.

Even though numerous lncRNAs were identified by using microarray and RNA-sequencing (RNA-seq) analyses in the testis (Bao et al. 2013, Sun et al. 2013) and it is known that testis lncRNAs account for the large percentage of entire lncRNAs (Necsulea et al. 2014), a small number of studies reported the function of testicular lncRNAs. *HongrES2* is suggested to be involved in the capacitation of sperm (Ni et al. 2011), and *Tsx* is shown to be critical in mouse spermatogenesis especially at the

spermatocyte stage (Anguera et al. 2011). *lncRNA-Tcam1* regulates immune-related genes in spermatocyte-derived cultured cells (Kurihara et al. 2017), and the lack of X-linked lncRNA, *Tslrn1*, shows significant reduction in the number of spermatozoa (Wichman et al. 2017). In *Drosophila,* it was found that some lncRNAs regulate spermatid individualization or formation of normal spermatids (Wen et al. 2016). These data strongly suggest the physiological significance of testicular lncRNAs in spermatogenesis, but more studies are obviously needed.

Here I identified two novel testis-specific lncRNAs, *lncRNA-HSVIII* and *Tesra* (*Tessp cluster lncRNA related to gene activation*), at the *Prss/Tessp* gene locus, and investigated their expression and function. In chapter 1, I obtained the data supporting that *lncRNA-HSVIII* was involved in multiple events during meiosis, and adjacent sequences of *lncRNA-HSVIII* functioned as the enhancer for the *Prss42/Tessp-2* gene. In chapter 2, I demonstrated the binding of *Tesra* to the *Prss42/Tessp-2* promoter and suggested that *Tesra* contributed to upregulation of *Prss42/Tessp-2* together with the enhancer surrounding *lncRNA-HSVIII*. These results pave the way for improving the understanding of molecular mechanism of spermatogenesis mediated by testicular lncRNAs.

Chapter 1 :

Identification and Characterization of *lncRNA-HSVIII* during Mouse Spermatogenesis and Enhancer Activity of its Flanking Sequences

SUMMARY

Spermatogenesis is regulated by many meiotic stage-specific genes, but how they coordinate the many individual processes is not fully understood. The *Prss/Tessp* gene cluster is located on the mouse chromosome 9F2-F3, and the three genes at this site (*Prss42/Tessp-2*, *Prss43/Tessp-3*, and *Prss44/Tessp-4*) are specifically activated during meiosis in pachytene spermatocytes. I searched for DNase I hyper-sensitive sites (HSs) and long noncoding RNAs (lncRNAs) at the *Prss/Tessp* locus to elucidate how they are activated. I found eight DNase I HSs, three of which were testis germ cell-specific at or close to the *Prss42/Tessp-2* promoter, and a testis-specific lncRNA, *lncRNA-HSVIII*, which was transcribed from a region adjacent to the *Prss42/Tessp-2* gene. *lncRNA-HSVIII* transcripts localized to nuclei of most pachytene spermatocytes and the cytosol of stage-X pachytene spermatocytes and spermatids. A 5.8-kb genome sequence, encompassing the entire *lncRNA-HSVIII* sequence and its flanking regions, significantly increased *Prss42/Tessp-2* promoter activity using a reporter gene assay, yet this construct did not change *lncRNA-HSVIII* expression, indicating that the elevated promoter activity was likely through enhancer activity. Indeed, both upstream and downstream regions of the *lncRNA-HSVIII* sequence significantly increased *Prss42/Tessp-2* promoter activity. In combination with a previous result identifying the direct interaction of a genomic region in the *lncRNA-HSVIII* locus with the *Prss42/Tessp-2* promoter in spermatocytes, my current data suggested that sequences adjacent to the lncRNA function as enhancers for the *Prss42/Tessp-2* gene.

INTRODUCTION

How genes are specifically activated at particular stages during spermatogenesis especially in primary spermatocytes—remains to be elucidated (Goldberg et al. 2010, Grimes 2004, Johnston et al. 2008, Ou et al. 2010, Shima et al. 2004, Wang et al. 2005). Three paralogous mouse genes *Prss42/Tessp-2*, *Prss43/ Tessp-3*, and *Prss44/Tessp-4*—constitute a gene cluster on mouse chromosome 9F2-F3, and are activated in primary spermatocytes at the late pachytene stage (Yoneda et al. 2013). These three genes, plus their homolog *Prss41/Tessp-1*, encode serine proteases that are crucial for meiotic progression at different stages (Yoneda & Kimura 2013; Yoneda et al. 2013). The functional significance and spermatocyte-specific expression of the *Prss/Tessp* genes suggest that this gene cluster would be an excellent model for studying the mechanisms of gene activation during meiosis.

Germ cell-specific genes were classically thought to be controlled solely by their proximal promoters, based on the identification of minimum promoters that were sufficient to activate genes in transgenic mice (Kehoe et al. 2008, Reddi et al. 2007). General transcription factors that are exclusively expressed in testicular germ cells are also known to play important roles in gene activation during meiosis (DeJong 2006, Goodrich & Tjian 2010). Yet, distal enhancer elements are being recognized for germ cell-specific gene activation. For example, the enhancer located between 4.8 kb and 1.3 kb of the transcriptional start site of the mouse *Ccna1* gene was necessary for full activation of this gene (Lele & Wolgemuth 2004b). Our group also recently found an enhancer for the mouse spermatocyte-specific *Tcam1* gene (Kurihara et al. 2014). Thus, gene expression during male meiosis is likely complicated by many layers of regulation.

Chromatin structure is essential to gene activation. DNase I hypersensitive sites (HS) are genomic regions with loose chromatin that are often marked with epigenetic modifications that facilitate the formation of active chromatin (Alabert & Groth 2012, Jenuwein & Allis 2001, Mercer et al. 2013) and are associated with promoters and enhancers (Cockerill 2011, Iwafuchi-Doi & Zaret

2014). Enhancers increase the transcription of their target genes by physically interacting with promoters, as revealed by the innovative chromosome conformation capture (3C) assay (Dekker et al. 2002, Tolhuis et al. 2002). Recently, enhancers were found to be controlled by long noncoding RNAs (lncRNAs) (Ponting et al. 2009, Umlauf et al. 2008, Wang & Chang 2011, Wilusz et al. 2009, Zaratiegui et al. 2007), which are extensively transcribed from the mammalian genome in organs including the mouse testis (Bao et al. 2013, Liang et al. 2014, Necsulea et al. 2014). In these examples, lncRNAs facilitated the dynamic chromatin rearrangements that allowed enhancers to contact promoter regions (Lai et al. 2013, Sanyal et al. 2012). In contrast to the known regulatory mechanisms of various tissue-specific genes (Arvey et al. 2012, Natarajan et al. 2012, Todeschini et al. 2014), little is known about how DNase I HSs and lncRNAs control specific gene activation in the testis during meiosis.

In this chapter, I assessed how the mouse *Prss/Tessp* gene cluster is activated. I found eight DNase I HSs at this locus as well as a testis-specific lncRNA, *lncRNA- HSVIII*, which was transcribed from a region adjacent to the *Prss42/Tessp-2* gene. In spermatocytes, the chromatin at *lncRNA-HSVIII* was reported to interact with the *Prss42/Tessp-2* promoter (Yoneda 2014), in which germ cell-specific DNase I HSs were present. Yet *lncRNA-HSVIII* transcription was unlikely to be related to the *Prss42/Tessp-2* gene regulation; instead, sequences adjacent to the lncRNA possessed *Prss42/Tessp-2* enhancer activity, thus revealing a novel mechanism of gene activation during male meiosis.

MATERIALS AND METHODS

Animals

The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University. C57BL6/Crj mice (CLEA Japan Inc., Tokyo, Japan) were maintained on 14hr light/10hr dark cycles at 25˚C, and fed with ad libitum food and water.

DNase I HS Mapping

Native germ cells were isolated from two adult mouse testes, and their purity was checked by marker gene expression, as previously described (Yoneda et al. 2013; Kurihara et al. 2014). The cells were lysed with 4 ml NP-40 lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) containing 1× proteinase inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Liver nuclei were collected by cutting and homogenizing a 0.1-g piece of liver in 4 ml NP-40 lysis buffer containing 1× proteinase inhibitor cocktail. The nuclei were collected by centrifugation, washed with 1 ml DNase I digestion buffer (0.1 M NaCl, 50 mM Tris-HCl [pH 7.5], 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM phenylmethylsulfonyl fluoride), suspended again in 625 μl DNase I digestion buffer, and divided into five aliquots. One hundred twenty-five microliters (125 μl) of DNase I digestion buffer was added to one aliquot, and mixed with 250 μl 2× Stop solution (20 mM Tris-HCl [pH 7.5], 100 mM EDTA, 600 mM NaCl, 1% SDS, 200 mg/ml proteinase K). The remaining four aliquots were mixed with 125 μl DNase I digestion buffer containing 40 U/ml DNase I (Takara, Ohtsu, Japan), and incubated at 37˚C for 15, 45, 90, and 180 sec. The reaction was stopped by adding 250 μl 2× Stop solution to each sample. All the five samples were incubated overnight at 55˚C to purify the genome DNA. The aliquot without DNase I digestion was used as the 0-sec sample time point.

The purified DNA was measured, and approximately 5-10 μg DNA was digested at 37˚C for 20 hr with restriction enzymes, *Hin*dIII, *Bam*HI, or *Eco*RI. The digested DNA was then purified by

phenol/chloroform isoamylalcohol (CIAA) extraction and ethanol precipitation. These samples were separated by electrophoresis on 0.7% agarose gels, and transferred to Hybond-N^+ membranes (GE Healthcare, Piscataway, NJ) for Southern blot.

Southern Blot

A 38-kb region encompassing the *Prss42/Tessp-2* gene was detected as two fragments following *Hin*dIII and *Bam*HI digestion (Fig. 1-1A). A 679-bp probe, which detected both fragments, was prepared by PCR amplification with ExTaq polymerase (Takara) and mouse genome DNA. Following PCR amplification, the products were subcloned into pBluescript II (Stratagene, La Jolla, CA), and their sequences were checked by DNA sequencing. I selected the clone that contained completely correct sequence for use as the probe; restriction digestion, agarose gel electrophoresis, and DNA purification from the gel was used to obtain the probe itself. The probe was ^{32}P-labeled and hybridized with the DNA-blotted membranes either at 65˚C in 0.5 M sodium phosphate [pH 7.2], 0.1 mM EDTA, 5% SDS, 1% bovine serum albumin, and 100 mg/ml herring sperm DNA or at 42˚C in 50% formamide, 5× Denhardt's solution, 5× SSPE, 1% SDS, and 100 mg/ml herring sperm DNA. The membranes were washed at 65˚C or 50˚C with 1× SSC/0.1% SDS, and the radioactive signal was detected by autoradiography using Kodak Biomax MR film. Fine mapping of the *Prss42/Tessp-2* promoter region was performed by digesting the DNase I-treated genomic DNA with *Eco*RI, and a 711-bp probe for this fragment was obtained by PCR as above (Fig. 1-1A). The primers for PCR reactions to prepare probes are listed in Table 2.

Reverse Transcription-PCR (RT-PCR) and Quantitative RT-PCR (qRT-PCR) Analyses

Total RNAs were extracted by ISOGEN II (Nippon gene, Tokyo, Japan) according to the manufacturer's instruction. After treatment with TurboDNase I (Ambion, Austin, TX), RNAs were reverse-transcribed into cDNAs using Superscript III (Invitrogen, Carlsbad, CA), according to the instruction. PCR was performed using ExTaq polymerase (Takara). qRT-PCR was performed using

KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) or Power SYBR Green Master Mix kit (Thermo Fisher Scientific, Kanagawa, Japan). Primer sequences are shown in Table2.

Rapid Amplification of cDNA Ends (RACE)

RACE analyses were performed as previously described (Kurihara et al. 2014, Matsubara et al. 2014) . For 5'RACE, reverse transcription was performed with RT-1 primer, and the first PCR was performed with GSP1 and the Abridged Anchor Primer. The second, nested PCR was performed using GSP2 and Abridged Universal Amplification Primer. For 3'RACE, the first and second PCRs were performed using GSP3 and GSP4, respectively, with 3-sites Adaptor Primer. All the products were subcloned into pBluescript II (Stratagene) using the TA-cloning method, and the sequences of at least ten subclones were determined for each experiment. Primer positions are indicated in Figure 1-2A, and their sequences are shown in Table 2.

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

The germ-cell fraction was prepared as above. Sertoli cells were obtained from 11-day-old testes by primary culture, as previously described (Yoneda & Kimura 2013). The Sertoli/Leydig-cell fraction was obtained from 6-month-old testes as follows: The testes were decapsulated and treated with 0.1% collagenase at 32˚C. The floating cells were collected and applied to a discontinuous Percoll gradient (20%, 37%, and 53%). After centrifugation, the cells between 37% and 53% were washed and collected as the Sertoli/Leydig-cell fraction.

Preparation of Subcellular Fractions

Testicular germ cells were lysed in NP-40 lysis buffer on ice for 10 min, and the lysate was centrifuged at 1,500 rpm for 5 min at 4˚C. The supernatant was used as the cytoplasmic fraction. The precipitates were washed with the same buffer 2-3 times, and the resulting pellet was used as the nuclear fraction.

In Situ Hybridization

Adult mouse testes were fixed for 3 hr at 4˚C with 4% paraformaldehyde in phosphate-buffered saline (PBS). Tissues were washed three times with PBS, and dissected into two pieces with a razor blade under a dissecting microscope. In situ hybridization with the TSA Plus system (Perkin Elmer, Waltham, MA) was performed according to a previously reported procedure (Kotani et al. 2013). Briefly, samples were dehydrated, embedded in paraffin, and cut into 7-μm thick sections. The sense and antisense RNA probes for *lncRNA-HSVIII* were synthesized with a digoxigenin (DIG) RNA labeling kit (Roche Molecular Biochemicals); I used a full-length sequence of *lncRNA-HSVIII* (2665 nucleotides) for labeling. After hybridization and washing, the sections were incubated for 30 min with anti-DIG-horseradish peroxidase antibody (1:500 dilution) (Roche Molecular Biochemicals). The reaction with tyramide-Cy3 was performed according to the manufacturer's instructions. After incubation with 10 mg/ml Hoechst33258 for 10 min, the samples were observed under an LSM5LIVE confocal microscope (Carl Zeiss, Oberkochen, Germany).

Plasmid Constructs

The *Prss42/Tessp-2* promoter sequence was amplified by PCR with mouse genome DNA using KOD polymerase (TOYOBO) and a primer pair listed in Table 2. The 1,636-bp product was blunted and phosphorylated, and inserted into a pGL-3 Basic vector (Promega Corporation, Madison, WI) at the blunted-*Hin*dIII site. I used the plasmid clone that contained no mutation in the promoter. The 5.8-kb fragment encompassing the *lncRNA-HSVIII* sequence was obtained by digestion of a mouse bacterial artificial chromosome clone, B6Ng01-306015 (RIKEN Bioresource Center), with *Eco*RI, and subcloned into pBluescript II (Stratagene). The fragment was further excised from the vector with *Eco*RI, blunted and phosphorylated, and inserted into the pGL-3 Basic vector containing the *Prss42/Tessp-2* promoter at the blunted *Bam*HI site. A 6.7-kb λ*Hin*dIII fragment was obtained from a λ*Hin*dIII marker, and inserted into the same vector at the blunted and dephosphorylated *Bam*HI site.

Full-length *lncRNA-HSVIII* sequence was first amplified separately by RT-PCR as a 2,562-bp 5'-half and a 259-bp 3'-half using primer pairs shown in Table 2. The two products were separately subcloned into pBluescript II KS(+) at the *Eco*RV site by the TA-cloning method, and confirmed by sequencing. Then, I digested both plasmids with *Bgl*II and *Sal*I, and inserted a 109-bp 3'-half into the plasmid containing a 2,556-bp 5'-half. After digestion of the plasmid, which contained the full-length of *lncRNA-HSVIII*, with *Bam*HI and *Sal*I, I ligated the *lncRNA-HSVIII* fragment into *Bam*HI/*Sal*I site of the pGL-3 Basic vector containing the *Prss42/Tessp-2* promoter. The digested *lncRNA-HSVIII* fragment was also blunted and phosphorylated, and inserted into the *Sma*I site of the modified plasmid.

Upstream and downstream sequences of *lncRNA-HSVIII* were obtained by digesting the pBluescript II vector containing the 5.8-kb fragment with *Sma*I/*Hinc*II and *Sac*I/ *Eco*RV, respectively. The fragments were blunted and phosphorylated, and inserted into the blunted *Bam*HI site of the pGL-3 Basic vector containing the *Prss42/Tessp-2* promoter.

Cell Culture, Transfection, and Reporter-Gene Assay

Hepa1-6 cells were cultured as previously described (Matsubara et al. 2010). P19TG1 cells were cultured with Eagle's minimum essential medium containing 10% fetal bovine serum and 2 mM L-glutamine, in the 37˚C incubator supplied with 5% CO_2. P19TG1 is an *Hprt*-deficient subline of the P19 embryonic carcinoma cell line (Mise et al. 1996), which was originally established from a teratocarcinoma by transplantation of an embryonic day-7.5 mouse embryo into the testis (McBurney & Rogers 1982).

I used the same molar quantity of DNA in each assay. For the reporter assay, Hepa1-6 cells were cultured in 24-well dishes and transfected with constructs using GeneJuice transfection reagent (Novagen, Madison, WI), according to the manufacturer's instructions. P19TG1 cells were cultured in 35-mm dishes and transfected using FuGENE-HD reagent (Promega Corporation). The day before transfection, 0.5×10^5 P19TG1 cells were seeded on a 35-mm dish; on the day of transfection, the cell

confluency was about 50–80 %, and were presented with complexes of various constructs formed with FuGENE-HD. The cells were collected 48 hr after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation).

The constructs I prepared above contained the firefly luciferase gene, and in each experiment, they were co-transfected with a pRL-CMV vector (Promega Corporation), by which the *Renilla* luciferase gene was driven by the CMV promoter. All the constructs were transfected as circular plasmids. Firefly luciferase activity was normalized to *Renilla* luciferase activity to adjust the transfection efficiency. The activity was further normalized to that of the cells transfected with a pGL3-Basic vector, and relative activity was calculated. The value from a pGL3-Basic vector was set to 1.0.

Statistical Analysis

Results were expressed as the average ± standard deviation (S.D.) of at least three independent experiments. Student's t-test and Dunnett's test were performed using Microsoft Excel statistical analysis functions (Microsoft Corporation, Redmond, WA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

DNase I HS Mapping at the Mouse *Prss/Tessp* Locus

DNase I HS mapping at the *Prss/Tessp* locus in testicular germ cells and liver cells was performed to identify cis-regulatory sequences that control the expression of these genes. The germ cell fraction was prepared as previously described (Yoneda et al. 2013); marker gene expression revealed that more than 70% of cells in the fraction were germ cells in this study. The liver was used as an irrelevant tissue that should not express any of these clustered genes. The nuclei from germ cells and liver cells were treated with DNase I, and DNAs were purified, digested with restriction enzymes, and subjected to Southern blot analysis.

I first examined two restriction fragments, resulting from HindIII or BamHI digestion, that covered a 38-kb region encompassing the *Prss42/Tessp-2* gene (Fig. 1-1A). By HindIII digestion, I detected an expected mother band at the position of 20 kb and three additional bands: two at 11.9 kb and 1.2 kb in both native germ cells and liver cells, and one at 9.9 kb only in the liver (Fig. 1-1B). These DNase I HSs corresponded to 13.3, 11.3, and 2.7 kb upstream of the *Prss42/Tessp-2* gene; I named them HSI, HSII, and HSIII (Fig. 1-1A and B). HSI was observed before DNase I treatment in the liver, possibly due to endogenous DNase activity. By BamHI digestion, I observed an expected mother band at 22 kb and four additional bands: two at 11.1 kb and 4.9 kb in both tissues, one at 6.7 kb only in the liver, and the other one at 3.9 kb only in native germ cells, although the signal intensity was different between the two cell types (Fig. 1-1C). These DNase I HSs corresponded to the promoter, gene body, and 3' flanking region of the *Prss42/Tessp-2* gene.

The presence of DNase I HSs at the *Prss42/Tessp-2* promoter is consistent with the active transcription of this gene in native germ cells. As I also detected a band corresponding to this promoter in liver cells by BamHI digestion (Fig. 1-1C, arrowhead), I performed fine mapping of this region by digestion with EcoRI. Southern blot analysis resulted in the detection of an expected mother band at 3.6 kb in both cells. In germ cells, three additional bands were observed at 2.4, 1.5, and 1.2 kb,

whereas no clear band was detected in the liver (Fig. 1-1D). The absence of DNase I HSs in the liver was not due to insufficient DNase I treatment because a similar extent of digestion was observed in the germ cell nuclei (Fig1-1E). Therefore, DNase I HSs in the EcoRI fragment were considered germ cell-specific. Mapping these DNase I HSs at the locus indicate that the 2.4-kb band corresponded to the signal at 3.9 kb by BamHI digestion, while the other two bands were presumed to be detected as one BamHI fragment at 4.9 kb. I named these DNase I HSs HSIV, HSV, and HSVI (Fig. 1-1C and D); the DNase I HSs located further downstream of them were named HSVII and HSVIII (Fig. 1-1C).

In sum, I identified eight DNase I HSs at the *Prss42/Tessp-2* locus: two of them (HSII and HSVII) were liver-specific, and three at or close to the *Prss42/ Tessp-2* promoter (HSIV, HSV, and HSVI) were present only in testicular germ cells (Fig. 1-1A).

Characterization of *lncRNA-HSVIII* Transcribed from an Adjacent Region of HSVIII

lncRNAs are important for the regulation of various genes (Rinn & Chang 2012), and there are many lncRNAs expressed in the testis (Bao et al. 2013; Sun et al. 2013; Liang et al. 2014). I therefore assessed noncoding transcription at the *Prss/Tessp* locus, which was suggested by testis transcriptome data (accession numbers: SRX135150, SRX135160, and SRX135162) that mapped to several intergenic regions at the *Prss/Tessp* locus. RT-PCR analyses revealed a novel lncRNA transcribed from the region adjacent to the *Prss42/Tessp-2* gene, starting near HSVIII; this segment was thus named *lncRNA-HSVIII* (Fig. 1-2A). This lncRNA was specifically detected by RT-PCR amplification of total RNA from whole testis (Fig. 1-2B).

I next examined which testicular cells expressed *lncRNA-HSVIII* by fractionating testicular cells. I prepared germ cell, Sertoli cell, and Sertoli/Leydig cell fractions, and then evaluated the expression of marker genes in these fractionations by RT-PCR (Fig. 1-2E). I detected *lncRNA-HSVIII* transcript in germ- and Sertoli/Leydig-cell fractions, but not in Sertoli cell fraction (Fig. 1-2C); the Sertoli-cell fraction did not contain any other cell types (Fig. 1-2E), implying that the origin of the signal in the Sertoli/Leydig-cell fraction was the Leydig cells. Therefore, *lncRNA-HSVIII* was expressed in germ

cells and Leydig cells, and the stronger signal in the germ cell fraction might represent its main role in meiosis. RT-PCR from nuclear and cytosolic RNA fractions of germ cells revealed its enrichment in the nucleus, with some present in the cytosol (Fig. 1-2D).

The full-length sequence of *lncRNA-HSVIII* was then determined by RACE analyses. One transcriptional start site was identified for *lncRNA-HSVIII* among 20 subclones using a 5'RACE. On the other hand, 3' RACE resulted in the identification of three transcriptional termination sites among 10 subclones. Interestingly, the repetitive sequence GAAAA was enriched in the 3' region, and the subclone had different numbers of this repeat: A final adenine of the 7th GAAAA sequence was present in seven subclones, whereas the 3rd and 10th GAAAA repeat was considered a transcriptional termination site in one and two subclones, respectively. By consensus, I determined that the 7th GAAAA represented the 3' end of *lncRNA-HSVIII*, resulting in a full-length *lncRNA-HSVIII* sequence of 2,665 nucleotides (Fig. 1-2A) plus a polyadenine tail (DDBJ/EMBL/GenBank accession number LC060751).

In situ hybridization with the highly sensitive tyramide signal amplification system was performed on adult testes to identify the localization of *lncRNA-HSVIII*. An abundance of punctate signal was detected with the *lncRNA-HSVIII* antisense probe, whereas few dots were observed with the sense probe (Fig. 1-3A and B). Consistent with RT-PCR result of fractionated testicular cells (Fig. 1-2C), *lncRNA-HSVIII* transcript was present in germ cells as well as the nuclei and cytosol of Leydig cells (Fig. 1-3C, arrows). Positive signals were observed at all seminiferous epithelial stages. Signal was observed in nuclei of some pachytene spermatocytes and in the cytosol of spermatids at stage V (Fig. 1-3D). Whereas signal remained stable in spermatids, the percentage found in the nuclei of pachytene spermatocytes increased at stage VIII (Fig. 1-3E). More spots were observed in nuclei of some pachytene spermatocytes or in the cytosol of others at stage X (Fig. 1-3F). Thus, *lncRNA-HSVIII* transcription begins in early pachytene spermatocytes, and is enhanced in late pachytene stages, when transcript localization transitions from the nucleus to the cytosol such that by the end of the pachytene stage, *lncRNA-HSVIII* transcripts are cytosolic where it remains in

spermatids.

Enhancer Activity in the Region Encompassing HSVIII and *lncRNA-HSVIII*

A previous study revealed the interaction of chromatin at *lncRNA-HSVIII* with upstream regions of the *Prss42/Tessp-2* gene specifically in spermatocytes (Yoneda et al. 2014). So I assumed that either *lncRNA-HSVIII* transcription or enhancer elements within or close to it are involved in activating the *Prss42/Tessp-2* gene.

Accordingly, I prepared a reporter construct containing a 5.8-kb EcoRI fragment fused with the luciferase gene driven by the *Prss42/Tessp-2* promoter (Fig. 1-4) to determine whether *lncRNA-HSVIII* transcription was related to *Prss42/Tessp-2* activation or the genomic sequence encompassing it functioned as an enhancer for *Prss42/Tessp-2*. This 5.8-kb fragment contained the entire sequence of *lncRNA-HSVIII* as well as its 1.9-kb promoter and 1.2-kb 3' sequence (Fig. 1-4A). For comparison, I also prepared a construct with a 6.7-kb λHindIII fragment (Fig. 1-4B and C). I used two established mouse cell lines, an embryonal carcinoma cell line, P19TG1, and a hepatic tumor line, Hepa1-6, because both endogenously expressed *Prss42/Tessp2* mRNA (data not shown).

The constructs were introduced separately into P19TG1 and Hepa1-6 cells, and luciferase activity was measured 2 days later. Activity for both cells was significantly higher in the construct with the murine 5.8-kb fragment than those without it or with λDNA (Fig. 1-4B and C). I measured the abundance of *lncRNA-HSVIII* in the transfected P19TG1 and Hepa1-6 cells by qRT-PCR to determine if the enhancement of *Prss42/Tessp-2* promoter activity was associated with *lncRNA-HSVIII* transcription. The amount of *lncRNA-HSVIII* transcript was not significantly different between the two constructs in P19TG1 cells (Fig. 1-5); in addition, I did not detect *lncRNA-HSVIII* transcript in Hepa1-6 cells transfected with them (data not shown). Thus, *lncRNA-HSVIII* was not transcribed from the transgene, which strongly suggests that the 5.8-kb sequence increased *Prss42/Tessp-2* promoter activity in P19TG1 and Hepa1-6 cells by functioning as an enhancer element.

I lastly assessed which region in the 5.8-kb sequence possessed enhancer activity by separating it into three regions: a 2.3-kb upstream region, a 2.7-kb *lncRNA-HSVIII* sequence, and 1.5-kb downstream region (Fig. 1-4A). The *lncRNA-HSVIII* sequence itself showed no enhancer activity in either P19TG1 cells or Hepa1-6 cells (Fig. 1-6A). In contrast, sequences upstream and downstream of *lncRNA-HSVIII* significantly increased *Prss42/Tessp-2* promoter activity in both cell types (Fig. 1-6B). Thus, sequences flanking *lncRNA-HSVIII* possess enhancer activity for the *Prss42/Tessp-2* gene, which suggests that genomic regions adjacent to *lncRNA-HSVIII* function as enhancers for the *Prss42/Tessp2* gene in spermatocytes

DISCUSSION

Many studies report that testicular germ cell-specific genes are controlled exclusively by their proximal promoters (Reddi et al. 2007), yet even if a promoter was sufficient for germ cell-specific gene activation, a transgene version of the locus is silenced or the expression level varies between transgenic lines (Li et al. 1998, Reddi et al. 1999, Robinson et al. 1989, Zambrowicz et al. 1993). These inconsistencies indicate that other elements, such as insulators and distal enhancers, are required for germ cell-specific gene regulation; indeed, the contribution of enhancers to gene expression during male meiosis was reported previously (Lele and Wolgemuth, 2004; Kurihara et al. 2014). My current data provide another example of potential enhancers of a spermatocyte-specific gene, and highlight how complicated gene activation can be during meiosis.

I first searched for DNase I HSs to identify cis-regulatory sequences that are involved in the activation of *Prss/Tessp* genes. Although eight DNase I HSs were identified, three were germ cell-specific and were positioned in or near the *Prss42/Tessp-2* promoter (Fig. 1-1). By analogy to the *Pgk2* and *Prm1* promoters, which containing DNase I HSs and are sufficient for their meiotic stage-specific activation (Kramer et al. 1998, Kumari et al. 1996, Martins & Krawetz 2007a, Peschon et al. 1987, 1989; Robinson et al. 1989), the presence of DNase I HSs in the *Prss42/Tessp-2* promoter supports its spermatocyte-specific activation. On the other hand, ubiquitously distributed DNase I HSs may be sufficient for tissue-specific gene expression, and enhancers are not necessarily associated with DNase I hypersensitivity. While the *Prss42/Tessp-2* promoter was obviously important for its spermatocyte-specific expression, I thought that distal cis-regulatory elements of this gene might also contribute to its specificity; indeed, I identified potential enhancer sequences adjacent to the *Prss42/Tessp-2* gene.

The presence of potential enhancers for *Prss42/Tessp-2*, but not the other two cluster genes, is consistent with the observation that *Prss42/Tessp-2* mRNA is the most abundant of the three cluster genes (Yoneda et al. 2013). Although it is unclear whether or not *Prss44/Tessp-4* and *Prss43/Tessp-3*

are regulated by their promoters, the interaction of potential enhancer elements only with the *Prss42/Tessp-2* promoter suggests different regulatory mechanisms are active at the cluster. For example, proximal promoters may be sufficient for activating *Prss44/ Tessp-4* and *Prss43/Tessp-3* whereas *Prss42/Tessp-2* expression involves several regulatory elements.

I tested two possible mechanisms of *Prss42/Tessp-2* activation: enhancer activity of genomic sequences encompassing *lncRNA-HSVIII*, and the activation by *lncRNA-HSVIII* transcription. Although they are not mutually exclusive, I attempted to distinguish between these possibilities by generating a reporter construct in which the luciferase gene, driven by the *Prss42/Tessp-2* promoter, was connected to a 5.8-kb fragment encompassing the entire *lncRNA-HSVIII* and its flanking sequences. This construct showed enhancer activity in two cell lines that express *Prss42/Tessp-2*, yet *lncRNA-HSVIII* was not transcribed from the transgene (Figs. 1-4 and 1-5), implying that the *Prss42/Tessp-2* promoter is regulated by an enhancer. I performed two additional experiments to exclude the lncRNA-mediated gene regulation model: First, I overexpressed *lncRNA-HSVIII* in Hepa1-6, P19TG1, and GC-2spd(ts) cells which were established from mouse primary spermatocytes (Hofmann et al. 1994), but mRNA abundance of *Prss/Tessp* cluster genes were not changed in any of the cells (data not shown). Second, overexpression of *lncRNA-HSVIII* did not increase *Prss42/Tessp-2* promoter activity in Hepa1-6 cells in a reporter gene assay (data not shown). Taken together with the observation that *lncRNA-HSVIII* transcript does not necessarily reside in the nuclei of late-stage pachytene spermatocytes that actively transcribe *Prss/Tessp* genes (Fig. 1-3), I concluded that *lncRNA-HSVIII* does not contribute to transcriptional activation of the cluster.

Even if *lncRNA-HSVIII* is not directly regulating expression of the *Prss/Tessp* cluster genes, it may contribute to meiotic regulation. Indeed, *lncRNA-HSVIII* transcript resides in nuclei of most pachytene spermatocytes, and its abundance dramatically increased at late pachytene stages (Fig. 1-3). Nuclear lncRNAs generally have one of the two functions: construction of nuclear structures, such as paraspeckles, or transcriptional regulation (Chen & Carmichael 2010). I presently do not know how *lncRNA-HSVIII* functions in pachytene spermatocytes, but because it was not consistently associated

with any specific nuclear structure, I speculate that this lncRNA participates in the transcriptional regulation of spermatocyte-specific genes. In spermatids and some pachytene spermatocytes at stage X, *lncRNA-HSVIII* transcript resides in the cytosol (Fig. 1-3F), suggesting its involvement in the regulation of RNA stability and translation (Atianand & Fitzgerald 2014, Guhaniyogi & Brewer 2001). Such a role in translational control is consistent with the level of translational arrest observed in early stages of primary spermatocytes, followed by their translation at later stages (Langford et al. 1993, Morales et al. 1991, Schäfer et al. 1995, Yan et al. 2010).

Genomic sequences adjacent to *lncRNA-HSVIII* were found to function as enhancers of *Prss42/Tessp-2* in embryonic carcinoma cells (P19TG1) and hepatic tumor cells (Hepa1-6), which endogenously expressed *Prss42/ Tessp-2*. Nevertheless, determining if the sequences adjacent to *lncRNA-HSVIII* really function as enhancers in native spermatocytes will be crucial. Many testis-specific genes are active in tumor cells (Simpson et al. 2005), suggesting that activation mechanisms of testis specific-genes are partly shared within the testis and in the cell lines used for our reporter studies. Further, the similar level of enhancer activity observed in two unrelated cell lines that expressed *Prss42/Tessp-2* mRNA (Figs. 1-4 and 1-6) strongly supports the model that the sequences flanking *lncRNA-HSVIII* do function as *Prss42/Tessp-2* enhancers in the testis. On the other hand, cell lines do not recapitulate processes that occur in native tissues. Hepa1-6 cells were derived from a mouse hepatoma (Darlington 1987, Darlington et al. 1980), and have been widely used to study biological events in the liver as well as for other purposes. P19TG1 is an *Hprt*-deficient subline of the P19 embryonic carcinoma cell line (Mise et al. 1996), as I described in the 'Materials and Methods' section. P19 cells are commonly used to study mechanisms of neurogenesis and myogenesis (Bain et al. 1994, van der Heyden & Defize 2003), but its derivation implies that these cells may share characteristics with spermatogenetic cells; indeed, Stra8, a factor for germ cell differentiation, was reported to be induced by retinoic acid in P19 cells (Oulad-Abdelghani et al. 1996). In my assay, some transcription factors that are commonly expressed in the testis and in the two cell lines I used might bind to the sequences flanking *lncRNA-HSVIII* and thereby enhance *Prss42/Tessp-2* promoter

activity.

Enhancer activity in the 5.8-kb fragment resided in two regions just upstream and downstream of *lncRNA-HSVIII* (Fig. 1-6), suggests that these regions cooperatively function to enhance *Prss42/Tessp-2* promoter activity a situation reminiscent of super-enhancers (Pott & Lieb 2014). Super-enhancers were first reported in mouse embryonic stem cells, and contain several enhancers, each located within 12.5 kb, that are utilized in a cell-specific manner (Whyte et al. 2013). In a super-enhancer, each enhancer interacts with the target promoter, resulting in the formation of complex chromatin structures. If both 3' and 5' sequences flanking *lncRNA-HSVIII* enhance *Prss42/Tessp-2* promoter activity in spermatocytes, they may interact with the promoter individually. Alternatively, the sequence 3' of *lncRNA-HSVIII* may function primarily as an enhancer whereas the sequence 5' of it may play an auxiliary role in gene regulation. It is unclear whether or not the flanking sequences to *lncRNA-HSVIII* are parts of a super-enhancer. Based on these data, however, we assume that at least the sequence 3' of *lncRNA-HSVIII* is required for activation of the *Prss42/Tessp-2* gene in spermatocytes during germ cell differentiation.

A gene cluster usually consists of several paralogues that originated from the duplication of an ancestral gene during evolution. The *Prss/Tessp* gene cluster contains three paralogues, likely from duplicated genes (Yoneda et al. 2013), yet the current data demonstrated that the *lncRNA-HSVIII* region interacts with the promoter of *Prss42/Tessp-2* but not the other cluster genes. Thus, each gene at the locus appear to be controlled by different elements contrary to most gene clusters, which are controlled as a group by a single cis-regulatory element, such as locus control region (Dean 2006, Festenstein & Kioussis 2000, Fraser & Grosveld 1998, Sproul et al. 2005). Even if cluster genes are expressed at different developmental stages or in different tissues, locus control regions regulate the entire cluster (Grosveld et al. 1987, Jones et al. 1995, Su et al. 2000). The distinct regulation of the individual *Prss/Tessp* genes thus provides new insight into how gene clusters can be regulated.

Chapter 2 :

A novel testis-specific long noncoding RNA, *Tesra*, activates the *Prss42*/*Tessp-2* gene during mouse spermatogenesis

SUMARRY

The progression of spermatogenesis is precisely controlled by meiotic stage-specific genes, but the molecular mechanism for activation of such genes is still elusive. Here I focused on a novel testis-specific long noncoding RNA (lncRNA), *Tesra*, transcribed at the *Prss/Tessp* gene cluster that was composed of *Prss42/Tessp-2*, *Prss43/Tessp-3*, and *Prss44/Tessp-4* genes specifically activated in pachytene spermatocytes. *Tesra* was 4,435 nucleotides long and transcribed from the 3' untranslated region of *Prss44/Tessp-4* present in both germ cell and Leydig cell fractions. Because *Tesra* was localized in nuclei of germ cells, I tested the possibility that it plays a role in the activation of *Prss/Tessp* genes. In hepatic tumor Hepa1-6 cells, transient overexpression of *Tesra* significantly activated the endogenous *Prss42/Tessp-2* expression and increased *Prss42/Tessp-2* promoter activity by a reporter gene assay. Consistently, I observed a high level of chromatin occupancy of the *Tesra* transcript at the *Prss42/Tessp-2* promoter region in testicular germ cells by chromatin isolation by RNA purification assay. These indicate that *Tesra* activates *Prss42/Tessp-2* promoter activity by binding to the promoter region. Furthermore, *Tesra* was found to co-function with an enhancer at the downstream of another lncRNA, *lncRNA-HSVIII*, for *Prss42/Tessp-2* activation, although they were not interdependent. Taken together, I identified a novel lncRNA, *Tesra*, and determined a function, which extend our understanding of the significance of testicular lncRNA in meiosis. Since an enhancer and a lncRNA are mostly located in adjacent regions to activate a target gene, my current data also provide the novel relationship between lncRNAs and enhancers.

INTRODUCTION

Spermatogenesis is the process to generate mature spermatozoa, and meiosis is a key step. During meiosis, diploid spermatogonia differentiate into primary spermatocytes, which divide twice with only one DNA replication, into haploid round spermatids (Eddy 2002, Hecht 1998). This process is tightly controlled by various factors, such as endocrine regulators (O'Shaughnessy 2014, Ramaswamy & Weinbauer 2016, Sinha Hikim & Swerdloff 1999) and testis-specific genes (Geng et al. 2016, Kashiwabara 2002, Kemphues et al. 1982), but the full picture of spermatogenesis is still unclear. To understand this mysterious event, tremendous efforts have been made, and in particular, researchers have studied the epigenetic regulation at each meiotic stage (Getun et al. 2016, Hammoud et al. 2014, Martins & Krawetz 2007b, Ontoso et al. 2014, Shirakata et al. 2014), the meiotic recombination (Ishiguro et al. 2014, Qiao et al. 2014), and the function of small RNAs (de Mateo & Sassone-Corsi 2014, Vourekas et al. 2016, Yadav & Kotaja 2014). Recently, a new factor, long noncoding RNA (lncRNA), was found to be potentially important in spermatogenesis.

lncRNA is a class of noncoding RNA over 200 nucleotides, and was found to be present in almost every tissue of every species by transcriptomic analyses performed at the beginning of the 21st century. Generally, lncRNAs are not evolutionarily conserved, when compared with protein-coding genes (Bergmann & Spector 2014, Rinn & Chang 2012, Ulitsky et al. 2011), and show tissue-specific expression (Necsulea et al. 2014), so they are considered to have some unique function in each tissue. Indeed, in mammalian somatic cells, lncRNAs are crucial regulators for chromatin remodeling, RNA processing, and construction of subnuclear structures. In particular, they control the protection of heart from stress-induced defects (Han et al. 2014, Hang et al. 2010), the tissue-specific alternative splicing in HeLa cells (Tripathi et al. 2010), and the construction of nuclear paraspeckles and morphogenesis in the mammary gland (Nakagawa et al. 2014, Standaert et al. 2014). By contrast, in germ cells, the function of lncRNAs have not been well studied, while so many lncRNAs are expressed during meiosis, as revealed by several high-throughput analyses (Iguchi et al. 2006, Liang

et al. 2014, Sun et al. 2013). To verify the significance of testicular lncRNAs, we need to reveal the function of more lncRNAs.

In chapter 1, I reported a novel testis-specific lncRNA, *lncRNA-HSVIII*, at the mouse *Prss/Tessp* gene cluster. The *Prss/Tessp* cluster is located on mouse chromosome 9F2-F3, and consists of three paralogous genes, *Prss42/Tessp-2*, *Prss43/Tessp-3*, *Prss44/Tessp-4*. They all are specifically activated in primary spermatocytes at the late pachytene stage, and *Prss42/Tessp-2* and *Prss43/Tessp-3* are involved in the progression of meiosis and the germ cell survival (Yoneda et al. 2013). *lncRNA-HSVIII* was transcribed in a testis-specific manner at the downstream of *Prss42/Tessp-2* gene, and interestingly, the localization was changed from nuclei of primary spermatocytes to cytosols of round spermatids during meiosis. Therefore, I suggested that *lncRNA-HSVIII* had multiple functions in spermatogenesis. On the other hands, during the study of *lncRNA-HSVIII*, I noticed that other intergenic regions might be transcribed in a testis-specific manner at the *Prss/Tessp* locus by analyzing transcriptomic data. Thus, the study of such novel lncRNAs would help us understand the function of more lncRNAs in spermatogenesis.

In this chapter, I identified another novel testis-specific lncRNA, *Tesra* (*Tessp cluster lncRNA related to gene activation*), at the *Prss/Tessp* locus. *Tesra* was expressed in both nuclei and cytosols of germ cells, and nuclear *Tesra* could increase *Prss42/Tessp-2* expression and its promoter activity. Consistently, *Tesra* bound to the *Prss42/Tessp-2* promoter for activation of the gene. In addition, I found that an enhancer at the downstream of *lncRNA-HSVIII* co-functioned with *Tesra* to activate the *Prss42/Tessp-2* gene promoter. These findings improve our understanding toward the biological significance of lncRNAs in spermatogenesis.

MATERIALS AND METHODS

Animals

C57BL6/Crj mice (CLEA Japan Inc., Tokyo, Japan) were maintained on regular 14 hr light/10 hr dark cycles at 25ºC, and fed with enough food and water. The experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

RT-PCR Analysis

Total RNA was extracted by ISOGEN II (Nippon gene, Tokyo, Japan) according to the manufacturer's instruction. After the treatment with TurboDNase (Life Technologies, Foster City, CA, USA), RNA was reverse-transcribed into cDNA using Superscript III (Life Technologies), based on the previously described method (Matsubara et al. 2010, 2014). PCR was performed by using ExTaq (Takara, Kusatsu, Japan). Primer sequences are shown in Table 3.

Isolation of Male Germ Cells, Sertoli Cells, and Leydig Cells

Isolation of male germ cells (Yoneda et al. 2013), Sertoli cells (Yoneda & Kimura 2013), and Leydig cells (Kurihara et al. 2014) were performed as previously described. Sertoli cells were harvested from 7-12 days old testes, and germ and Leydig cells were from adult testes.

Preparation of Subcellular Fractions

The cells were lysed in NP-40 lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, pH 7.5) on ice for 10 min, and the lysates were centrifuged at 1,500 rpm for 5 min at 4°C. The resulting supernatant was used as the cytoplasmic fraction. The precipitates were washed with NP-40 lysis buffer 2-3 times, and the resulting pellet was used as the nuclear fraction.

qRT-PCR Analysis

cDNAs were prepared as above. PCRs were performed by using Power SYBR Green PCR Master Mix (Life Technologies) or KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan), based on the previously described method (Kurihara et al. 2014, Matsubara et al. 2010). The relative expression levels of lncRNAs or protein-coding genes were normalized to endogenous *Aip* mRNA. Primer sequences are shown in Table 3.

5'RACE and 3' RACE

5'RACE and 3'RACE were performed as previously described (Matsubara et al. 2014). For 5'RACE, cDNA was generated by using the total RNA from adult mouse testes and a gene specific primer (GSP1) for reverse transcription. After the addition of oligodeoxycytidine by terminal deoxynucleotidyl transferase (Takara), the first PCR was performed with GSP2 and the abridged anchor primer. The second PCR was performed using GSP3 and the abridged universal amplification primer.

For 3'RACE, reverse transcription was performed using the oligo$(dT)_{20}$ primer connected to an adaptor sequence. The first and second PCRs were performed using GSP4 and GSP5, respectively, with the adaptor primer.

All primer sequences are shown in Table 3. Each final PCR product was subcloned into a pBluescript II vector by the TA-cloning method, and their sequences were confirmed by DNA sequencing analysis.

Plasmid Constructs

Tesra-OE: The full-length of *Tesra* was obtained by RT-PCR with mouse testis cDNA and subcloned into pBluescript II KS(+). After the DNA sequence was confirmed, the plasmid was digested with *Bam*HI and *Eco*RI, and the *Tesra* fragment was inserted into a pcDNA3.1(+) vector which was cut by *Bam*HI and *Eco*RI.

T-2pro-luc: This construct contained the luciferase gene driven by the *Prss42/Tessp-2* promoter

as I mentioned in chapter 1.

T-2pro-luc-up and T-2pro-luc-down: These constructs contained a 2.3-kb upstream or a 1.5-kb downstream sequence of *lncRNA-HSVIII* at 3' of the luciferase gene in T-2pro-luc. They both were generated in chapter 1.

Tesra-tet-on: The full-length of *Tesra* was obtained and subcloned into pBluescript II KS(+) as above but in an opposite direction. This plasmid was digested with *Spe*I and *Xho*I, and the *Tesra* fragment was inserted into a pPBhCMV*1-cHA-pA vector which was cut by *Spe*I and *Xho*I (Murakami et al. 2016). The pPBhCMV*1-cHA-pA plasmid and other vectors required to establish the Tet-on system were kindly gifted by Dr. Kazuhiro Murakami (Murakami et al. 2016).

Cell Culture and Transfection

Hepa1-6 cells were cultured as previously described (Kurihara & Kimura 2015, Kurihara et al. 2014). Transfection into Hepa1-6 cells was conducted according to the protocol of GeneJuice reagent (Merck, Darmstadt Germany).

Overexpression of *Tesra*

Tesra-OE was transfected into Hepa1-6 cells by using Genefectine Transfection Reagent (Genetrone Biotech, Seoul, South Korea). I started the selection 24 hours after the transfection by treating the cells with 0.5-1.5 mg/ml G418 for 3 days. After the selection, the cells were dissolved in ISOGEN II and total RNAs were purified. These RNAs were used for qRT-PCR.

In case of the Tet-on system, I transfected Tesra-tet-on, pPBCAGrtTA-IN which bore a neomycin resistance gene, and pPyCAG-Pbase into Hepa1-6 cells. Twenty four hours later, I selected the successfully transfected cells by treating with 2.0 mg/ml G418 for 24 days and established the stable cell line, in which the *Tesra* expression could be induced by the addition of Doxycycline (Dox). Then, T2pro-luc, T-2pro-luc-up or T-2pro-luc-down was transfected into these stable cells, and one day later, 1.0 μg/ml Dox was added. Twenty four hours after the addition of Dox, the cells were used

for luciferase assay.

Luciferase Assay

Luciferase assay was conducted according to the protocol of Dual-Luciferase Reporter Assay System (Promega).

Chromatin Isolation by RNA Purification (ChIRP) Assay

Twenty six antisense oligo probes for *Tesra* were designed by using singlemoleculefish.com 18 (http://singlemoleculefish.com) as indicated in Table 4, and biotinylated at the 3' end. Twenty million male germ cells were prepared from 21-22 day old mice as above. The cells in PBS were spun at 800-1,000 rpm for 4 min, resuspended in 1% glutaraldehyde, and crosslinked for 10 min at room temperature on a rotator. The cross-linking reaction was quenched with 1/10th volume of 1.25 M glycine at room temperature for 5 min. After washing the cells with PBS, I added 10× volume of the Lysis Buffer (final concentration of 50 mM Tris-Cl pH 7.0, 10 mM EDTA, 1% SDS) supplemented with phenylmethanesulfonyl fluoride, 1× Protease Inhibitor (Wako, Osaka, Japan), and RNasin ® Plus (Promega, Madison, WI, USA) to each tube and lysed them. Then, we sonicated the cell lysate to shear the chromatin for 10 sec, 15 times by using Ultrasonic Disruptor UD-201 (TOMY, Tokyo, Japan). The sonication was continued until the cell lysate was no longer turbid. The sample was spun at 15,000 rpm for 10 min at 4°C, and the supernatant was collected into another tube. 1% of this sample were taken as Input. Next, the sample was hybridized with the combined oligo probes at the final concentration of 100 pmol/ml in Hybridization Buffer (750 mM NaCl, 1% SDS, 50 mM Tris-Cl pH 7.0, 1 mM EDTA, 15% formamide) at 37°C for 4 hrs with shaking. The streptoavidin beads (GE healthcare, Uppsala, Sweden) (100 µl per 100 pmol probe) were washed with Lysis Buffer three times, and resuspended in Lysis Buffer supplemented with phenylmethanesulfonyl fluoride, Proteinase Inhibitor, and RNasin ® Plus. After the hybridization reaction, 100 µl beads were added to the tube and incubated at 37°C for 30 min with shaking. After the shaking, the beads were washed with 1 ml

wash buffer (2× NaCl and Sodium citrate (SSC) and 0.5% SDS) five times, and resuspended them in 1 ml wash buffer. I removed 100 µl and set aside for RNA isolation, and the rest of 900 µl was used for DNA extraction. Each tube was centrifuged at 4,000 rpm for 1 min at room temperature, and the beads were collected. For RNA sample, I added ISOGENII and purified total RNAs for qRT-PCR. For DNA sample, 50 µl DNA Elution Buffer (50 mM $NaHCO_3$, 1% SDS) was added with 1.0 µl 10 mg/ml RNase A (Nacalai, Kyoto, Japan) and 0.3 µl 10 U/µl RNase H (Bio Academia, Osaka, Japan), and incubated at 37°C for 30 min with shaking. After the shaking, I centrifuged the tubes at 4,000 rpm, and the supernatant was collected in a new tube. This process was repeated twice. 15 µl 10 mg/ml Proteinase K (Nacalai) was added into the combined supernatants and incubated at 50 °C for 45 min. After the incubation, The DNA was extracted by an organic solvent phenol and chloroform, and collected by an alcohol precipitation. Finally quantitative PCR (qPCR) was performed by using the collected DNA. Primers for this qPCR are shown in Table 3. The relative chromatin enrichment of each region was obtained by calculating the ratio of ChIRP sample to Input sample, and the occupancy level was further normalized to the value at the *Rec8* promoter, which was set to 1.0. As a control, I also prepared the RNase(+) sample that was treated with RNase in hybridization reaction to decline *Tesra* transcripts.

Statistical Analysis

Results were expressed as the average ± standard deviation (S.D.) of at least three independent experiments and analyzed by Student's *t*-test, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or Dunnett's test. P value less than 0.05 was considered statistically significant.

RESULTS

A Novel lncRNA, *Tesra*, is Transcribed from the Mouse *Prss/Tessp* Locus

In chapter 1, I found many reads mapped to intergenic regions at the mouse *Prss/Tessp* locus specifically in the testis by transcriptome data (accession numbers: SRX135150, SRX135160, and SRX135162). This suggested the presence of testis-specific noncoding transcripts at this locus, and especially, the intergenic region between *Prss44/Tessp-4* and *Prss43/Tessp-3* genes contained more reads. Thus, I performed RT-PCR with mouse testes by using a primer set of Tsera-ss and Tesra-as, and a 2,890-nucleotide sequence was successfully amplified (Fig. 2-1A). I named this lncRNA *Tesra* and decided to determine the full-length.

To define 5'- and 3'-ends of *Tesra*, I performed RACE analyses. By 5'RACE experiments, I obtained one specific band after the second PCR, and five different transcriptional start sites (TSSs) were detected by sequencing of 17 subclones. Because 10 subclones of them indicated an adenine as TSS, I defined it as a major TSS of *Tesra* (Fig 2-1B). By 3'RACE, I amplified one band in the second PCR, and detected a single TTS at 4,236 bp downstream of *Prss44/Tessp-4* as a result of sequencing of 10 subclones (Fig. 2-1C). Consequently, the full-length of *Tesra* was 4,435 bp. This lncRNA contained no intron, and was presumed to have the poly(A)-tail because some subclones contained the chain of adenine nucleotides longer than oligo(dT). Interestingly, 199 bp of the full-length of *Tesra* overlapped with the *Prss44/Tessp-4* gene. Taken together, a novel lncRNA, *Tesra*, was transcribed from the *Prss/Tessp* locus, and I determined the full-length sequence.

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The Expression Pattern of *Tesra*

I examined the tissue specificity of *Tesra* using eight mouse tissues by RT-PCR, and *Tesra* showed testis-specific expression as with the *Prss/Tessp* genes (Fig. 2-2A) (Yoneda et al. 2013). I next investigated the cell type specificity of *Tesra* in the testis, which contained three kinds of cells: germ cells, Sertoli cells, and Leydig cells. We prepared three fractions that were enriched in each type

of cells. The Sertoli cell fraction was prepared by primary culture and basically contained no other types of cells. The germ cells and Leydig cells were prepared from adult testes, and the most contaminants were Sertoli cells and germ cells, respectively. *Tesra* was detected in germ cell and Leydig cell fractions by RT-PCR, but not in the Sertoli cell fraction (Fig. 2-2B), which suggested that germ cells and/or Leydig cells expressed *Tesra*.

Then, I prepared total RNA from the nuclear or cytoplasmic fraction of germ cells to investigate the subcellular localization of *Tesra*. RT-PCR showed that *Tesra* was present in both nuclear and cytoplasmic fractions (Fig. 2-2C). I also investigated the expression pattern in the mouse testis at postnatal days 7, 14, 21, 28, and 56. *Tesra* showed expression from the postnatal day 7, and during day 14-21, the expression level was dramatically increased to become a peak (Fig. 2-2D). The timing of an increase in *Tesra* transcription coincided with the activation of *Prss/Tessp* expression at the pachytene spermatocyte stage (Yoneda et al. 2013). These data indicated that *Tesra* was expressed specifically in testicular germ cells and possibly in Leydig cells and localized in both cytoplasm and nucleus.

Prss42/Tessp-2 **Gene Activation by** ***Tesra***

Because *Tesra* transcription was increased at a similar timing to *Prss/Tessp* gene activation and the transcript was localized in nuclei, *Tesra* was possibly involved in the activation of *Prss/Tessp* genes during meiosis. To verify this possibility, I used a mouse hepatic tumor cell line, Hepa1-6, because *Prss/Tessp* genes were endogenously expressed in this cell (Fig. 2-3A). I first performed the overexpression of *Tesra*. I prepared two constructs, Tesra-OE and a control vector, and transfected each into Hepa1-6 cells. The successfully transfected cells were selected with G418, and the endogenous expression of the cluster genes were evaluated by qRT-PCR. The *Tesra* expression was significantly increased in the cells with *Tesra*-OE compared with the control (Fig 2-3B). The overexpression significantly increased *Prss42/Tessp-2* expression but did not affect *Prss43/Tessp-3* and *Prss44/Tessp-4* genes (Fig. 2-3C), which suggested that *Tesra* was involved in *Prss42/Tessp-2*

gene activation.

I then investigated whether *Tesra* increased *Prss42/Tessp-2* promoter activity by transient luciferase reporter gene assay. I prepared the construct in which the luciferase gene was driven by a 1.6-kb *Prss42/Tessp-2* promoter (T-2pro-luc), and co-transfected it with Tesra-OE into Hepa1-6 cells. As a result of measurement of luciferase activity 48 hours later, *Prss42/Tessp-2* promoter activity was significantly increased, compared to co-transfection with the control vector (Fig. 2-3D). These data supported that *Tesra* contributed to the activation of *Prss42/Tessp-2* gene by increasing *Prss42/Tessp-2* promoter activity.

Tesra Occupancy at the *Prss42/Tessp-2* Promoter

If *Tesra* actually increased *Prss42/Tessp-2* promoter activity *in vivo*, the *Tesra* transcript should directly interact with *Prss42/Tessp-2* promoter. So I investigated the occupancy of *Tesra* at the *Prss/Tessp* locus. To reveal the genome occupancy of *Tesra* at the *Prss42/Tessp-2* promoter, ChIRP assay was conducted with male germ cells from 21-22 days old mice (Chu et al. 2011, 2012), because the expression level of *Tesra* was the highest at these stages (Fig. 2-2D). I hybridized the sonicated chromatin with biotinylated tiling oligos, and collected and purified the bound DNAs for qPCR. As a control, I prepared the RNase(+) sample that was treated with RNase in hybridization reaction to decline *Tesra* transcripts. I amplified genomic regions at the *Prss/Tessp* locus, and *Rec8* and *B2m* promoters were examined as negative controls. The occupancy level at the *Rec8* promoter was set to 1.0.

Most regions showed no significant difference in genomic occupancy compared with *Rec8* promoter, but the *Prss42/Tessp-2* promoter showed significantly higher level of genome occupancy (Fig. 2-4). Although the level was not significantly different from *Rec8* promoter, the region transcribed into *Tesra* exhibited a little higher occupancy. This was considered to be artifact by direct interaction of tiling oligos with genome DNA, as evidenced by the control data (RNase+) showing a clear peak at this region (Fig. 2-4). In contrast, no peak was observed at the *Prss42/Tessp-2* promoter

in the RNase(+) sample, indicating the actual binding of *Tesra* to this region. Collectively, *Tesra* interacted with the *Prss42/Tessp-2* promoter at the *Prss/Tessp* locus in mouse germ cells, which strongly suggested that a testis-specific lncRNA, *Tesra*, increased *Prss42/Tessp-2* promoter activity via interaction with the promoter region.

Co-activation of *Prss42/Tessp-2* Promoter Activity by an Enhancer and *Tesra*

In chapter 1, I reported that upstream and downstream sequences of *lncRNA-HSVIII* possessed enhancer activity for *Prss42/Tessp-2* (Fig. 1-6). I investigated whether *Tesra* was functionally related with these enhancers for *Prss42/Tessp-2* activation. To solve this issue, I established *Tesra*-inducible stable cells by using Hepa1-6 cells. This stable cell line was established by co-transfection of Tesra-tet-on with other required vectors and the selection with G418. The Dox treatment and qRT-PCR verified that *Tesra* transcription was about 80-fold increased by the culture for 24 hours (Fig. 2-5C).

I transfected T-2pro-luc, T-2pro-luc-up, or T-2pro-luc-down into the *Tesra*-inducible stable cell line, and added Dox 24 hours after the transfection. After another 24 hours, I measured luciferase activity. Without Dox, I confirmed enhancer activity of both upstream and downstream sequences for *Tessp-2* promoter (Fig. 2-5B). With T-2pro-luc-up, promoter activity significantly increased about 1.3 folds, and with T-2pro-luc-down, promoter activity significantly increased about 1.7 folds (Fig. 2-5B, white bars). These results were consistent with chapter 1 (Fig. 1-6). By the addition of Dox, I observed further increase in *Tessp-2* promoter activity with T-2pro-luc-down; the activity was significantly increased 2.2 folds (Fig. 2-5B, black bars of T-2pro-luc vs T-2pro-luc-down). In contrast, promoter activity was not significantly changed in the cells with T-2pro-luc-up by the Dox treatment (Fig. 2-5B, black bars of T-2pro-luc vs T-2pro-luc-up). These results indicated that *Tesra* could co-operatively but independently increase *Prss42/Tessp-2* promoter activity with the downstream enhancer.

Discussion

In this chapter, I identified a novel mouse testis-specific lncRNA, *Tesra*. I revealed that *Tesra* expression was detected in both germ cell and Leydig cell fractions by RT-PCR (Fig. 2-2B). Because major contaminants in the Leydig cell fraction was germ cells, it was possible that the signal in this fraction might be derived from germ cells. However, we detected the *Tesra* signal in 7-day-old testes, suggesting the expression in somatic cells, especially Leydig cells. In addition, *Tesra* was found to be localized in both cytosol and nucleus of germ cells. Many lncRNAs were reported to be localized in both nuclei and cytosols and play different roles dependent on their localization (Ayupe et al. 2015, Cabili et al. 2015). While nuclear lncRNAs construct subcellular structure or regulate transcription (Lai et al. 2013, Nakagawa et al. 2014, Yang et al. 2013a), cytosolic lncRNAs were involved in post transcriptional regulation such as RNA translation (Abdelmohsen et al. 2014, Tran et al. 2016) and RNA stability (Gong & Maquat 2011, Kretz et al. 2012), or function as precursors of miRNAs (Dey et al. 2014, Legnini et al. 2014). Therefore, *Tesra* likely plays multiple roles in the testis, and this time I found a function of the transcriptional activation of the *Prss42/Tessp-2* gene.

Here I focused on the function of *Tesra* in nuclei of pachytene spermatocytes. Because many nuclear lncRNAs were reported to control the transcription of their neighboring genes and the *Prss/Tessp* cluster genes were activated in pachytene spermatocytes, I hypothesized that *Tesra* contributed to their transcriptional activation. In this chapter, I obtained the following evidence that supported this hypothesis. First, *Tesra* significantly interacted with the chromatin at the *Prss42/Tessp-2* promoter in germ cells by ChIRP assay (Fig. 2-4). This confirmed the *in vivo* interaction of the *Tesra* transcript with the *Prss42/Tessp-2* promoter. Second, *Tesra* significantly increased the endogenous *Prss42/Tessp-2* gene expression by transient overexpression in Hepa1-6 cells (Fig. 2-3C). Third, the transient overexpression of *Tesra* also increased *Prss42/Tessp-2* promoter activity by transient reporter gene assay (Fig. 2-3D). These indicate that *Tesra* contributes to transcriptional activation of the *Prss42/Tessp-2* gene by enhancing promoter activity.

In the nucleus, lncRNAs affect the expression of protein-coding genes and ncRNAs in cis (Deocesano-Pereira et al. 2014, Wang et al. 2011, Yu et al. 2008) or in trans (Kino et al. 2010, Rinn et al. 2007). Cis-acting lncRNA is defined that it targets to genes on the same chromosome as the lncRNA and never affects genes on other chromosomes. On the other hand, trans-acting lncRNA can affect genes on different chromosomes. Although *Tesra* contributed to activation of a neighboring gene, *Prss42/Tessp-2*, on the same chromosome, I showed that *Tesra* could activate the *Prss42/Tessp-2* transcription in trans by the result of the transient overexpression and reporter gene assay (Fig. 2-3). Several trans-acting lncRNAs were reported to target three or more genes that were located at different chromosomes (Kaneko et al. 2014, Zhang et al. 2014). Given that *Tesra* was detected by 30 cycles of PCR by RT-PCR with germ cell nuclei (Fig. 2-2C), it was expressed at a relatively high level as lncRNA and presumed to target more genes other than *Prss42/Tessp-2*. Therefore, I assume that *Tesra* regulates the transcription of several genes in trans.

How does *Tesra* increase *Prss42/Tessp-2* promoter activity? Many trans-acting lncRNAs recruit histone modification enzymes or transcription factors to chromatin regions of target genes. For example, *MEG3* facilitates the recruitment of PRC2 and JARID2 to a subset of target genes to mark the chromatin with H3K27me3 (Kaneko et al. 2014). *H19* lncRNA directly represses *Igf2*, *Slc38a4*, and *Peg1* by recruiting the methyl-CpG–binding domain protein 1 (MBD1) to differentially methylated regions of three imprinted genes (Monnier et al. 2013). Similarly to these lncRNAs, *Tesra* may recruit transcription factors or histone modification enzymes to the *Prss42/Tessp-2* promoter region. In this case, it is possible that a part of *Tesra* sequence recognizes the DNA sequence of *Prss42/Tessp-2* promoter as a guide, as reported in some studies demonstrating the formation of DNA-RNA triplex with regulatory regions of target genes (Mondal et al. 2015, O'Leary et al. 2015). Alternatively, *Tesra* may function as a scaffold which interacts with multiple protein complexes, as reported for *HOTTIP* associated with the WDR5/MLL1 chromatin-modifying complex (Cheng et al. 2015) and *HOTAIR* with PRC2 and the LSD1/CoREST/REST complex (Bhan & Mandal 2015). Further studies will be required for understanding the detailed molecular mechanism concerning the

function of *Tesra*.

lncRNAs often collaborate with enhancers to activate their target genes. For instance, one class of lncRNA, enhancer-derived RNAs (eRNAs) are unidirectionally or bidirectionally transcribed at enhancer regions, and mediate the histone modification for active chromatin (Hah et al. 2013, Zhu et al. 2013b) and the chromatin looping between the enhancer and promoter (Hsieh et al. 2014, Sanyal et al. 2012). Some lncRNAs are not transcribed at enhancers but still affect the looping between distal enhancers and promoters (Xiang et al. 2014). In any case, these functional lncRNAs regulate the transcription of their target genes through the action on enhancers, and therefore, lncRNAs are mostly considered to facilitate enhancer activity. In contrast, the transcriptional activation of *Prss42/Tessp-2* by *Tesra* and the enhancer appeared to be different from such examples. I reported enhancer activity of upstream and downstream sequences of *lncRNA-HSVIII* in chapter 1, but *Tesra* was transcribed at a distal region from these enhancers. In addition, my present data indicated that both *Tesra* and two enhancers could increase *Prss42/Tessp-2* promoter activity independently in luciferase assay (Fig. 2-5B). Each enhancer could individually activate the promoter, and *Tesra* also activated *Prss42/tessp-2* transcription in the absence of the enhancers. The downstream enhancer and *Tesra* just co-worked for increase in *Prss42/Tessp-2* promoter activity when they co-existed (Fig. 2-5B). These indicate that *Tesra* and the enhancer are not interdependent. To best of my knowledge, there have been no report showing such relationship between lncRNAs and enhancers, so my data revealed a novel gene regulatory mechanism concerning lncRNA and enhancer.

Out of the two potential enhancers, *Tesra* collaborated only with the downstream enhancer because the induction of *Tesra* transcription additively increased *Prss42/Tessp-2* promoter activity with the downstream sequence but not with upstream (Fig. 2-5B). Consistently, it was actually the downstream sequence that interacted with *Prss42/Tessp-2* promoter by 3C assay (Yoneda 2014). Therefore, the two enhancers may function redundantly; the downstream enhancer may mainly function in the transcriptional activation of *Prss42/Tessp-2*, but when it is impaired, the gene may be activated by the upstream enhancer. Such a mechanism may be evolved due to the extreme

importance of spermatogenesis for animals.

Recent studies identified numerous lncRNAs in each stage of spermatogenesis, but their functions are still unclear. At present, only a few mammalian lncRNAs are found to be critical in male germ cell development, especially in meiotic stage. For instance, *testis-specific X-linked* (*Tsx*), which is specifically expressed in pachytene spermatocytes, is crucial for progression of meiosis, and possibly regulates *Tsix* during spermatogenesis (Anguera et al. 2011). Downregulation of *mrhl* RNA is required for activation of Wnt signaling and the entry of spermatogonia into meiosis (Akhade et al. 2016). *R53* containing a SINE-B1 motif is expressed mainly in nuclei of spermatocytes, and the reduction causes abnormal upregulation of expression of *Tnp1*,*Tnp2,* and *Prm1* genes, resulting in impairment of meiosis (Nakajima et al. 2017). While these data indicate the significance of lncRNAs in spermatogenesis, more lncRNAs have to be functionally analyzed when we consider the large number of lncRNAs identified in the testis. Obviously, I successfully added a function of a testicular lncRNA in spermatogenesis, especially meiosis, by the present data.

In summary, I revealed the expression pattern and the function of a novel lncRNA, *Tesra*. *Tesra* contributes to the activation of *Prss42/Tessp-2* gene, and due to the significant role of this gene in progression of meiosis from secondary spermatocytes to round spermatids (Yoneda et al. 2013), this lncRNA plays a significant role in spermatogenesis. My current data also revealed a meiosis-related function of testis-specific lncRNA in spermatocytes, and proposed the novel relationship between lncRNA and enhancer. These conduce toward the understanding how spermatogenesis is regulated and provide a new insight into the lncRNA function.

GENERAL DISCUSSION

The activation mechanism of Prss42/Tessp-2 gene

In this study, I revealed that two factors contribute to *Prss42/Tessp-2* transcriptional activation (Fig. 3). First, a downstream region of *lncRNA-HSVIII*, which interacted with the *Prss42/Tessp-2* promoter by looping out the intervening region, could function as an enhancer. Second, a lncRNA, *Tesra*, which was transcribed at the downstream of *Prss44/Tessp-4*, bound to the chromatin at the *Prss42/Tessp-2* promoter to increase its promoter activity. Both factors activated *Prss42/Tessp-2* transcription through increasing *Prss42/Tessp-2* promoter activity.

From my current and our laboratory's previous data, I propose the following mechanism for *Prss42/Tessp-2* gene activation during meiosis. In differentiated type B spermatogonia, germ cell-specific DHSs at the *Prss42/Tessp-2* promoter are probably formed, because they were reported to be observed at many poised promoters prior to transcriptional activation (Anguita et al. 2004, Kimura et al. 2007, Litt et al. 2001, Szutorisz et al. 2005). By this DHSs formation, the chromatin can be easily transferred to the active state. When type B spermatogonia divide into primary spermatocytes, the chromatin at the *Prss42/Tessp-2* promoter begins to interact with the *lncRNA-HSVIII* region at its 3' region and the *Prss42/Tessp-2* transcription is initiated (Yoneda 2014). This interaction is maintained and even strengthen in secondary spermatocytes, and the downstream sequence of *lncRNA-HSVIII* comes to physically contact the *Prss42/Tessp-2* promoter to increase its transcriptional activity. In the meanwhile, probably at the similar timing of the chromatin interaction, a lncRNA, *Tesra*, is expressed (Fig. 2-2D) and binds to the *Prss42/Tessp-2* promoter directly or indirectly to positively affect its promoter activity (Figs. 2-3, 2-4).

As I described in general introduction, meiotic genes had been classically considered to be fully activated only by their proximal promoters, but it is not necessarily the case. Given that no studies elucidated the molecular mechanism of spermatocyte-specific gene activation in details like this study, my current data made a significant contribution to understanding spermatogenesis in the field of reproduction and gene regulation.

The novel relationship between an enhancer and a lncRNA

My data indicate that the downstream enhancer and *Tesra* can co-function in the activation of *Prss42/tessp-2* promoter, but the mechanism was different from those reported in other studies in several aspects. Previous studies reported that lncRNAs were required for the histone modification at enhancers for gene activation (Hah et al. 2013, Zhu et al. 2013b) or for the chromatin looping between enhancers and promoters (Hsieh et al. 2014, Sanyal et al. 2012, Xiang et al. 2014). These lncRNAs regulate their target genes through the activation of enhancers or the formation of chromatin looping, and thus, they are mostly considered to facilitate enhancer activity. In addition, in those examples, lncRNAs were transcribed from adjacent regions of enhancers. In contrast, the transcriptional activation of *Prss42/Tessp-2* by *Tesra* and the downstream enhancer is different, in that 1) *Tesra* was transcribed from a distant region of the enhancer, and 2) the enhancer activity was not dependent on *Tesra* activity because they could independently affect *Prss42/Tessp-2* promoter activity (Fig. 2-5B).

However, we need more study to reveal the detailed mechanism of the *Prss42/Tessp-2* gene activation by the enhancer and *Tesra*. In most cases, transcription factors binding to promoters and lncRNA-binding proteins mediate functions of enhancers and lncRNAs (Bell et al. 2015, Kadonaga et al. 1986, Kim et al. 2016, Liu et al. 2015), so the *Prss42/Tessp-2* gene is probably activated by such proteins as well as *Tesra* and the enhancer. The identification of those protein factors will be my future scope. In any case, the gene activation mechanism and relationship between a lncRNA and an enhancer revealed by this study provide novel insights into the gene regulatory mechanism and the function of lncRNAs not only in male germ cells but also in other types of cells.

The regulation of the Prss/Tessp gene cluster

Even though the *Prss42/Tessp-2* gene belongs to the *Prss/Tessp* gene cluster, in which the three genes are specifically activated at the same timing in primary spermatocytes, my data indicate that each *Prss/Tessp* gene is regulated by different mechanisms from each other. So far, several gene clusters have been investigated in details for their transcriptional activation. For instance, the human

HOXD gene cluster is regulated by a lncRNA, *HOTAIR*, which was transcribed at the *HOXC* locus on a different chromosome (Rinn et al. 2007). The *β-globin* gene cluster and the *hGH* gene cluster are both controlled by locus control region (LCR), which contains tissue- and developmental stage-specific enhancers and lncRNAs that are essential for the cluster gene activation (Kim & Dean 2012, Tsai et al. 2016, Xu et al. 2010). These examples show that the genes composed of a cluster are generally regulated by a single element, although LCR contains several factors. In contrast, the *Prss/Tessp* cluster genes are likely regulated by different molecular mechanisms; the *Prss42/Tessp-2* gene needs an enhancer and *Tesra*, whereas *Prss44/Tessp-4* and *Prss43/Tessp-3* genes are unlikely controlled by them because neither chromatin interaction nor lncRNA occupancy was observed.

Cluster genes activation by LCR often involves the chromatin looping. The binding of key transcription factors or the noncoding transcription at enhancer sequences within LCR led to the chromatin looping to make the interaction between LCR and cluster genes (Kim et al. 2015). In these examples, the lncRNA transcription is necessary for the enhancer action. Furthermore, enhancer-derived RNAs, a class of lncRNA, were repeatedly reported to be necessary for the formation or the maintenance of chromatin looping (Xiang et al. 2014, Yang et al. 2013b). By contrast, *Tesra* was presumably not required for the chromatin looping between the *Prss42/tessp-2* promoter and the downstream enhancer, considering that *Tesra* was transcribed at a distant region from both the promoter and enhancer. This is consistent with my hypothesis that the enhancer and *Tesra* act on *Prss42/Tessp2* transcription independently. Notably, the gene activation by lncRNAs that are not dependent on enhancer functions have not been reported. My present study thus revealed a novel regulatory mechanism of the gene cluster, and this is specific to meiotic germ cells at present, but it is possible that these mechanisms can also be observed in somatic cells.

The function of lncRNAs during spermatogenesis

Many lncRNAs were identified by using microarray and RNA-sequencing (RNA-seq) analyses in the testis (Bao et al. 2013, Sun et al. 2013), and it is known that testis lncRNAs account for the

large percentage of entire lncRNAs (Necsulea et al. 2014). Nevertheless, only a small number of studies reported the function of testicular lncRNAs. Obviously, more studies are needed, and I identified two novel lncRNAs, *lncRNA-HSVIII* and *Tesra*, at the *Prss/Tessp* gene locus and investigated their functions.

As for *lncRNA-HSVIII*, I found that it was not involved in the transcriptional activation of *Prss/Tessp* cluster genes. On the other hands, I detected the interesting change of its localization during meiosis; in pachytene spermatocytes, *lncRNA-HSVIII* transcripts were present in nuclei, while in round spermatids, they were found in cytosols. A similar localization change was reported for mouse *R53* lncRNA that was shown to play an important role in the progression of meiosis in *in vitro* spermatogenesis (Nakajima et al. 2017). *lncRNA-HSVIII* may have critical functions in spermatogenesis like this lncRNA. In addition, dot-like signals in nuclei of pachytene spermatocytes by *in situ* hybridization may indicate that *lncRNA-HSVIII* is related to the transcriptional regulation of multiple gene loci at this stage (Fig. 1-3). Although further analyses will be necessary for revealing the function of *lncRNA-HSVIII*, I assume that it has multiple functions in meiosis and/or spermatogenesis.

In contrast, I determined a function of *Tesra*, as a positive regulatory factor for one of the cluster genes, *Prss42/Tessp-2*, by enhancing *Prss42/Tessp-2* promoter activity. Considering that the *Prss42/Tessp-2* gene is activated at the late pachytene stage (Yoneda et al. 2013) and *Tesra* is activated in primary spermatocytes (Fig. 2-2D), this lncRNA is likely to regulate this gene in primary spermatocytes. Only a few studies reported the function of lncRNA at this stage of spermatogenesis. *Tsx* is a X-linked lncRNA that is robustly expressed in pachytene spermatocytes, and its knock out mice exhibited the meiotic arrest at the pachytene spermatocyte stage (Anguera et al. 2011). *R53* is a lncRNA containing a B1 SINE sequence, and its knock down suggested that it was required for the meiosis progression into spermatids (Nakajima et al. 2017). Our group identified *lncRNA-Tcam1* that was expressed at a high level in spermatocytes, but it was suggested to regulate immune-related genes for immune response in the testis (Kurihara et al. 2017). In addition, in male germ cells, target genes

and the binding to their promoters were demonstrated for few lncRNAs, except for mouse *mrhl* RNA, which binds to the Sox8 promoter to activate the gene in spermatogonium-derived GC-1spg cell (Akhade et al. 2016). Therefore, my current study identified the target gene of a lncRNA and showed its binding to the promoter, extending the knowledge of lncRNA in meiosis, and is valuable in the field of lncRNAs in spermatogenesis.

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REFERENCES

Abdelmohsen K, Panda AC, Kang MJ, Guo R, Kim J, et al. 2014. NAR Breakthrough Article 7SL RNA represses p53 translation by competing with HuR. *Nucleic Acids Res.* 42(15):10099–111

Akhade VS, Dighe SN, Kataruka S, Rao MRS. 2016. Mechanism of Wnt signaling induced down regulation of mrhl long non-coding RNA in mouse spermatogonial cells. *Nucleic Acids Res.* 44(1):387–401

Alabert C, Groth A. 2012. Chromatin replication and epigenome maintenance. *Nat. Rev. Mol. Cell Biol.* 13(3):153–67

Ameres SL, Zamore PD. 2013. Diversifying microRNA sequence and function. *Nat. Rev. Mol. Cell Biol.* 14(8):475–88

Anguera MC, Ma W, Clift D, Namekawa S, Kelleher III RJ, Lee JT. 2011. *Tsx* Produces a Long Noncoding RNA and Has General Functions in the Germline, Stem Cells, and Brain. *PLoS Genet*. 7(9):e1002248

Anguita E, Hughes J, Heyworth C, Blobel GA, Wood WG, Higgs DR. 2004. Globin gene activation during haemopoiesis is driven by protein complexes nucleated by GATA-1 and GATA-2. *EMBO J.* 23(14):2841–52

Arvey A, Agius P, Noble WS, Leslie C. 2012. Sequence and chromatin determinants of cell-type-specific transcription factor binding. *Genome Res.* 22(9):1723–34

Atianand MK, Fitzgerald KA. 2014. Long non-coding RNAs and control of gene expression in the immune system. *Trends Mol. Med.* 20(11):623-31

Ayupe AC, Tahira AC, Camargo L, Beckedorff FC, Verjovski-Almeida S, Reis EM. 2015. Global analysis of biogenesis, stability and sub-cellular localization of lncRNAs mapping to intragenic regions of the human genome. *RNA Biol.* 12(8):877–92

Bain G, Ray WJ, Yao M, Gottlieb DI. 1994. From embryonal carcinoma cells to neurons: the P19

pathway. *Bioessays*. 16(5):343–48

Bao J, Wu J, Schuster AS, Hennig GW, Yan W. 2013. Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. *Biol. Reprod.* 89(5):107

Bell RJA, Rube HT, Kreig A, Mancini A, Fouse SD, et al. 2015. The transcription factor GABP selectively binds and activates the mutant TERT promoter in cancer. *Science.* 348(6238):1036–39

Bergmann JH, Spector DL. 2014. Long non-coding RNAs: Modulators of nuclear structure and function *Curr. Opin. Cell Biol.* 26:10-8

Bergmann M, Nashan D, Nieschlag E. 1989. Pattern of compartmentation in human seminiferous tubules showing dislocation of spermatogonia. *Cell Tissue Res.* 256(1):183–90

Bhan A, Mandal SS. 2015. LncRNA HOTAIR: A master regulator of chromatin dynamics and cancer *Biochim Biophys Acta.* 1856(1):151-64

Bult CJ, Eppig JT, Blake JA, Kadin JA, Richardson JE, et al. 2016. Mouse Genome Database 2016. *Nucleic Acids Res.* 44(D1):D840–7

Cabili M, Trapnell C, Goff L, Koziol M, Tazon-Vega B, et al. 2011. Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes Dev.* 25(18):1915–27

Cabili MN, Dunagin MC, McClanahan PD, Biaesch A, Padovan-Merhar O, et al. 2015. Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. *Genome Biol.* 16(1):20

Chen L-L, Carmichael GG. 2010. Decoding the function of nuclear long non-coding RNAs. *Curr. Opin. Cell Biol.* 22(3):357–64

Chen SR, Liu YX. 2015. Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling *Reproduction* 6(35):37012-27

Cheng Y, Jutooru I, Chadalapaka G, Corton JC, Safe S. 2015. The long non-coding RNA HOTTIP enhances pancreatic cancer cell proliferation, survival and migration. *Oncotarget*. 6(13):10840–

52

Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. 2011. Genomic Maps of Long Noncoding RNA Occupancy Reveal Principles of RNA-Chromatin Interactions. *Mol. Cell*. 44(4):667–78

Chu C, Quinn J, Chang HY. 2012. Chromatin Isolation by RNA Purification (ChIRP). *J. Vis. Exp.* (61):

Cockerill PN. 2011. Structure and function of active chromatin and DNase I hypersensitive sites. *FEBS J.* 278(13):2182–2210

Darlington GJ. 1987. Liver Cell Lines. *Methods Enzymol.* 151(C):19–38

Darlington GJ, Bernhard HP, Miller RA, Ruddle FH. 1980. Expression of liver phenotypes in cultured mouse hepatoma cells. *J. Natl. Cancer Inst.* 64(4):809–19

de Mateo S, Sassone-Corsi P. 2014. Regulation of spermatogenesis by small non-coding RNAs: Role of the germ granule Semin. *Cell Rev. Biol.* 29:84-92

Dean A. 2006. On a chromosome far, far away: LCRs and gene expression. *Trends Genet.* 22(1):38–45

DeJong J. 2006. Basic mechanisms for the control of germ cell gene expression. *Gene*. 366(1):39–50

Dekker J, Rippe K, Dekker M, Kleckner N. 2002. Capturing chromosome conformation. *Science*. 295(5558):1306–11

Deocesano-Pereira C, Amaral MS, Parreira KS, Ayupe AC, Jacysyn JF, et al. 2014. Long non-coding RNA INXS is a critical mediator of BCL-XS induced apoptosis. *Nucleic Acids Res.* 42(13):8343–55

Dey BK, Pfeifer K, Dutta A. 2014. The H19 long noncoding RNA gives rise to microRNAs miR-675-3p and miR-675-5p to promote skeletal muscle differentiation and regeneration. *Genes Dev.* 28(5):491–501

Dym M, Fawcett DW. 1970. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol. Reprod.* 3(3):308–26

Eddy EM. 2002. Male germ cell gene expression. *Recent Prog. Horm. Res.* 57:103–28

Erkkilä K, Henriksén K, Hirvonen V, Rannikko S, Salo J, et al. 1997. Testosterone regulates apoptosis in adult human seminiferous tubules in vitro. *J. Clin. Endocrinol. Metab.* 82(7):2314–21

Festenstein R, Kioussis D. 2000. Locus control regions and epigenetic chromatin modifiers. *Curr. Opin. Genet. Dev.* 10(2):199–203

Fraser P, Grosveld F. 1998. Locus control regions, chromatin activation and transcription. *Curr. Opin. Cell Biol.* 10(3):361–65

Geng Q, Ni L, Ouyang B, Hu Y, Zhao Y, Guo J. 2016. A Novel testis-specific gene, *Ccdc136* , is required for acrosome formation and fertilization in mice. *Reprod. Sci.* 23(10):1387–96

Getun VI., Wu Z, Fallahi M, Ouizem S, Liu Q, et al. 2016. Functional roles of acetylated histone marks at mouse meiotic recombination hotspots. *Mol. Cell. Biol.* 37(3)

Goldberg E, Eddy EM, Duan C, Odet F. 2010. LDHC: the ultimate testis-specific gene. *J. Androl.* 31(1):86–94

Gong C, Maquat LE. 2011. lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3′ UTRs via Alu elements. *Nature*. 470(7333):284–88

Goodrich JA, Tjian R. 2010. Unexpected roles for core promoter recognition factors in cell-type-specific transcription and gene regulation. *Nat. Rev. Genet.* 11(8):549–58

Grimes SR. 2004. Testis-specific transcriptional control. *Gene*. 343(1):11–22

Grosveld F, van Assendelft GB, Greaves DR, Kollias G. 1987. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell*. 51:975–85

Guhaniyogi J, Brewer G. 2001. Regulation of mRNA stability in mammalian cells *Gene* 265(1-2):11-23

Hah N, Murakami S, Nagari A, Danko CG, Lee Kraus W. 2013. Enhancer transcripts mark active estrogen receptor binding sites. *Genome Res.* 23(8):1210–23

Hammoud SS, Low DHP, Yi C, Carrell DT, Guccione E, Cairns BR. 2014. Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. *Cell Stem*

Cell. 15(2):239–53

Han P, Li W, Lin CH, Yang J, Shang C, et al. 2014. A long noncoding RNA protects the heart from pathological hypertrophy. *Nature*. 514(7520):102–6

Hang CT, Yang J, Han P, Cheng HL, Shang C, et al. 2010. Chromatin regulation by Brg1 underlies heart muscle development and disease. *Nature*. 466(7302):62–67

Hecht NB. 1998. Molecular mechanisms of male germ cell differentiation. *Bioessays*. 20(7):555–61

Heward JA, Lindsay MA. 2014. Long non-coding RNAs in the regulation of the immune response *Trends Immunol.* 35(9):408-19

Hofmann MC, Hess RA, Goldberg E, Millán JL. 1994. Immortalized germ cells undergo meiosis in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 91(12):5533–37

Hsieh C-L, Fei T, Chen Y, Li T, Gao Y, et al. 2014. Enhancer RNAs participate in androgen receptor-driven looping that selectively enhances gene activation. *Proc. Natl. Acad. Sci. U. S. A*. 111(20):7319–24

Iguchi N, Tobias JW, Hecht NB. 2006. Expression profiling reveals meiotic male germ cell mRNAs that are translationally up- and down-regulated. *Proc. Natl. Acad. Sci. U. S. A.* 103(20):7712–17

Ishiguro KI, Kim J, Shibuya H, Hernández-Hernández A, Suzuki A, et al. 2014. Meiosis-specific cohesin mediates homolog recognition in mouse spermatocytes. *Genes Dev.* 28(6):594–607

Iwafuchi-Doi M, Zaret KS. 2014. Pioneer transcription factors in cell reprogramming. *Genes Dev.* 28(24):2679–92

Jenuwein T, Allis CD. 2001. Translating the histone code. *Science*. 293(5532):1074–80

Johnsson P, Lipovich L, Grandér D, Morris VK. 2014. Evolutionary conservation of long non-coding RNAs; sequence, structure, function. *Biochim. Biophys. Acta - Gen. Subj.* 1840(3):1063–71

Johnston DS, Wright WW, Dicandeloro P, Wilson E, Kopf GS, Jelinsky SA. 2008. Stage-specific gene expression is a fundamental characteristic of rat spermatogenic cells and Sertoli cells. *Proc. Natl. Acad. Sci. U. S. A.* 105(24):8315–20

Jones BK, Monks BR, Liebhaber SA, Cooke NE. 1995. The human growth hormone gene is regulated

by a multicomponent locus control region. *Mol. Cell. Biol.* 15(12):7010–21

Kadonaga JT, Jones KA, Tjian R. 1986. Promoter-specific activation of RNA polymerase II transcription by Sp1. *Trends Biochem. Sci.* 11(1):20-23

Kaneko S, Bonasio R, Saldaña-Meyer R, Yoshida T, Son J, et al. 2014. Interactions between JARID2 and Noncoding RNAs Regulate PRC2 Recruitment to Chromatin. *Mol. Cell*. 53(2):290–300

Kashiwabara S, Noguchi J, Zhuang T, Ohmura K, Honda A, et al. 2002. Regulation of spermatogenesis by testis-specific, cytoplasmic poly(A) polymerase TPAP. *Science* 298(5600):1999–2002

Kehoe SM, Oka M, Hankowski KE, Reichert N, Garcia S, et al. 2008. A conserved E2F6-binding element in murine meiosis-specific gene promoters. *Biol. Reprod.* 79(5):921–30

Kemphues KJ, Kaufman TC, Raff R A, Raff EC. 1982. The testis-specific beta-tubulin subunit in Drosophila melanogaster has multiple functions in spermatogenesis. *Cell*. 31(December):655–70

Kim A, Dean A. 2012. Chromatin loop formation in the β-globin locus and its role in globin gene transcription. *Mol. Cells* 34(1):1-5

Kim J, Abdelmohsen K, Yang X, De S, Grammatikakis I, et al. 2016. LncRNA OIP5-AS1/cyrano sponges RNA-binding protein HuR. *Nucleic Acids Res.* 44(5):2378–92

Kim YW, Lee S, Yun J, Kim A. 2015. Chromatin looping and eRNA transcription precede the transcriptional activation of gene in the β-globin locus. *Biosci. Rep.* 35(2):1–8

Kimura AP, Sizova D, Handwerger S, Cooke NE, Liebhaber SA. 2007. Epigenetic activation of the human growth hormone gene cluster during placental cytotrophoblast differentiation. *Mol. Cell. Biol.* 27(18):6555–68

Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. 2010. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. *Sci. Signal.* 3(107):ra8

Kotani T, Yasuda K, Ota R, Yamashita M. 2013. Cyclin B1 mRNA translation is temporally controlled through formation and disassembly of RNA granules. *J. Cell Biol.* 202(7):1041–55

Kramer J, McCarrey J, Djakiew D, Krawetz S. 1998. Differentiation: the selective potentiation of

chromatin domains. *Development*. 125(23):4749–55

Kretz M, Siprashvili Z, Chu C, Webster DE, Zehnder A, et al. 2012. Control of somatic tissue differentiation by the long non-coding RNA TINCR. *Nature*. 493(7431):231–35

Kumari M, Stroud JC, Anji A, McCarrey JR. 1996. Differential appearance of DNase I-hypersensitive sites correlates with differential transcription of Pgk genes during spermatogenesis in the mouse. *J. Biol. Chem.* 271(24):14390–97

Kung JTY, Colognori D, Lee JT. 2013. Long noncoding RNAs: Past, present, and future. *Genetics*. 193(3):651–69

Kurihara M, Kimura AP. 2015. Characterization of the human TCAM1P pseudogene and its activation by a potential dual promoter-enhancer: Comparison with a protein-coding mouse orthologue. *FEBS Lett.* 589(4):540–47

Kurihara M, Otsuka K, Matsubara S, Shiraishi A, Satake H, Kimura AP. 2017. A Testis-Specific Long Non-Coding RNA, lncRNA-Tcam1, Regulates Immune-Related Genes in Mouse Male Germ Cells. *Front. Endocrinol. (Lausanne).* 8(299):1–12

Kurihara M, Shiraishi A, Satake H, Kimura AP. 2014. A conserved noncoding sequence can function as a spermatocyte-specific enhancer and a bidirectional promoter for a ubiquitously expressed gene and a testis-specific long noncoding RNA. *J. Mol. Biol.* 426(17):3069–93

Lai F, Orom UA, Cesaroni M, Beringer M, Taatjes DJ, et al. 2013. Activating RNAs associate with Mediator to enhance chromatin architecture and transcription. *Nature*. 494(7438):497–501

Langford KG, Zhou Y, Russell LD, Wilcox JN, Bernstein KE. 1993. Regulated expression of testis angiotensin-converting enzyme during spermatogenesis in mice. *Biol. Reprod.* 48(6):1210–18

Lee RC, Feinbaum RL, Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*. 75(5):843–54

Legnini I, Morlando M, Mangiavacchi A, Fatica A, Bozzoni I. 2014. A Feedforward regulatory loop between HuR and the long noncoding RNA linc-MD1 controls early phases of myogenesis. *Mol. Cell*. 53(3):506–14

Lele KM, Wolgemuth DJ. 2004. Distinct regions of the mouse cyclin A1 gene, Ccna1, confer male germ-cell specific expression and enhancer function. *Biol. Reprod.* 71(4):1340–47

Li H, MacLean G, Cameron D, Clagett-Dame M, Petkovich M. 2009. Cyp26b1 expression in murine sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. *PLoS One*. 4(10):

Li S, Zhou W, Doglio L, Goldberg E. 1998. Transgenic mice demonstrate a testis-specific promoter for lactate dehydrogenase, LDHC. *J. Biol. Chem.* 273(47):31191–94

Liang M, Li W, Tian H, Hu T, Wang L, et al. 2014. Sequential expression of long noncoding RNA as mRNA gene expression in specific stages of mouse spermatogenesis. *Sci. Rep.* 4:5966

Litt MD, Simpson M, Gaszner M, Allis CD, Felsenfeld G. 2001. Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. *Science*. 293(5539):2453–55

Liu S, Zhu J, Jiang T, Zhong Y, Tie Y, et al. 2015. Identification of lncRNA MEG3 Binding Protein Using MS2-Tagged RNA Affinity Purification and Mass Spectrometry. *Appl. Biochem. Biotechnol.* 176(7):1834–45

Martins RP, Krawetz S a. 2007b. Decondensing the protamine domain for transcription. *Proc. Natl. Acad. Sci. U. S. A.* 104(20):8340–45

Matsubara S, Kurihara M, Kimura AP. 2014. A long non-coding RNA transcribed from conserved non-coding sequences contributes to the mouse prolyl oligopeptidase gene activation. *J. Biochem.* 155(4):243–56

Matsubara S, Takahashi T, Kimura AP. 2010. Epigenetic patterns at the mouse prolyl oligopeptidase gene locus suggest the CpG island in the gene body to be a novel regulator for gene expression. *Gene*. 465(1–2):17–29

McBurney MW, Rogers BJ. 1982. Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol.* 89(2):503–8

Mercer TR, Edwards SL, Clark MB, Neph SJ, Wang H, et al. 2013. DNase I – hypersensitive exons

colocalize with promoters and distal regulatory elements. *Nat. Publ. Gr.* 45(8):852–59

Mise N, Sado T, Tada M, Takada S, Takagi N. 1996. Activation of the inactive X chromosome induced by cell fusion between a murine EC and female somatic cell accompanies reproducible changes in the methylation pattern of the Xist gene. *Exp. Cell Res.* 223(2):193–202

Mondal T, Subhash S, Vaid R, Enroth S, Uday S, et al. 2015. MEG3 long noncoding RNA regulates the TGF-β pathway genes through formation of RNA–DNA triplex structures. *Nat. Commun.* 6:7743

Monnier P, Martinet C, Pontis J, Stancheva I, Ait-Si-Ali S, Dandolo L. 2013. H19 lncRNA controls gene expression of the Imprinted Gene Network by recruiting MBD1. *Proc. Natl. Acad. Sci. U. S. A.* 110(51):20693–98

Morales CR, Kwon YK, Hecht NB. 1991. Cytoplasmic localization during storage and translation of the mRNAs of transition protein 1 and protamine 1, two translationally regulated transcripts of the mammalian testis. *J. Cell Sci.* 100 (Pt 1):119–31

Morlando M, Ballarino M, Fatica A. 2015. Long Non-Coding RNAs: New Players in Hematopoiesis and Leukemia. *Front. Med.* 2:23

Murakami K, Günesdogan U, Zylicz JJ, Tang WWC, Sengupta R, et al. 2016. NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers. *Nature*. 529(7586):1–22

Nakagawa S, Shimada M, Yanaka K, Mito M, Arai T, et al. 2014. The lncRNA Neat1 is required for corpus luteum formation and the establishment of pregnancy in a subpopulation of mice. *Development*. 141(23):4618–27

Nakajima R, Sato T, Ogawa T, Okano H, Noce T. 2017. A noncoding RNA containing a SINE-B1 motif associates with meiotic metaphase chromatin and has an indispensable function during spermatogenesis. *PLoS One*. 12(6):

Natarajan A, Yardimci GG, Sheffield NC, Crawford GE, Ohler U. 2012. Predicting cell-type-specific gene expression from regions of open chromatin. *Genome Res.* 22(9):1711–22

Necsulea A, Soumillon M, Warnefors M, Liechti A, Daish T, et al. 2014. The evolution of lncRNA

repertoires and expression patterns in tetrapods. *Nature*. 505(7485):635–40

Ng KW, Anderson C, Marshall EA, Minatel BC, Enfield KSS, et al. 2016. Piwi-interacting RNAs in cancer: emerging functions and clinical utility. *Mol. Cancer*. 15(1):5

Ni MJ, Hu ZH, Liu Q, Liu MF, Lu MH, et al. 2011. Identification and characterization of a novel non-coding rna involved in sperm maturation. *PLoS One*. 6(10):

O'Leary VB, Ovsepian SV, Carrascosa LG, Buske FA, Radulovic V, et al. 2015. PARTICLE, a triplex-forming long ncRNA, regulates locus-specific methylation in response to low-dose irradiation. *Cell Rep.* 11(3):474–85

O'Shaughnessy PJ. 2014. Hormonal control of germ cell development and spermatogenesis. *Semin. Cell Dev. Biol.* 29:55–65

Ontoso D, Kauppi L, Keeney S, San-Segundo PA. 2014. Dynamics of DOT1L localization and H3K79 methylation during meiotic prophase I in mouse spermatocytes. *Chromosoma*. 123(1–2):147–64

Ou C-M, Lin S-R, Lin H-J, Luo C-W, Chen Y-H. 2010. Exclusive expression of a membrane-bound Spink3-interacting serine protease-like protein TESPL in mouse testis. *J. Cell. Biochem.* 110(3):620–29

Oulad-Abdelghani M, Bouillet P, Décimo D, Gansmuller A, Heyberger S, et al. 1996. Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *J. Cell Biol.* 135(2):469–77

Pang KC, Frith MC, Mattick JS. 2006. Rapid evolution of noncoding RNAs: Lack of conservation does not mean lack of function *Trends Genet.* 22(1):1-5

Pasquinelli AE, Ruvkun G. 2002. Control of developmental timing by microRNAs and their targets. *Annu. Rev. Cell Dev. Biol.* 18(1):495–513

Pelletier RM. 2011. The blood-testis barrier: The junctional permeability, the proteins and the lipids

Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. 1987. Spermatid-specific expression of protamine 1 in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 84(15):5316–19

Peschon JJ, Behringer RR, Palmiter RD, Brinster RL. 1989. Expression of mouse protamine 1 genes in Transgenic Mice. *Ann. N. Y. Acad. Sci.* 564:186–97

Ponting CP, Oliver PL, Reik W. 2009. Evolution and functions of long noncoding RNAs. *Cell*. 136(4):629–41

Pott S, Lieb JD. 2014. What are super-enhancers? *Nat. Genet.* 47(1):8–12

Qiao H, Prasada Rao HBD, Yang Y, Fong JH, Cloutier JM, et al. 2014. Antagonistic roles of ubiquitin ligase HEI10 and SUMO ligase RNF212 regulate meiotic recombination. *Nat. Genet.* 46(2):194–99

Quinn JJ, Chang HY. 2016. Unique features of long non-coding RNA biogenesis and function

Ramaswamy S, Weinbauer GF. 2016. Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone. *Spermatogenesis*. 4(2):e996025

Reddi PP, Flickinger CJ, Herr JC. 1999. Round spermatid-specific transcription of the mouse SP-10 gene is mediated by a 294-base pair proximal promoter. *Biol. Reprod.* 61(5):1256–66

Reddi PP, Urekar CJ, Abhyankar MM, Ranpura SA. 2007. Role of an insulator in testis-specific gene transcription. *Ann. N. Y. Acad. Sci.* 1120:95–103

Rinn JL, Chang HY. 2012. Genome regulation by long noncoding RNAs. *Annu. Rev. Biochem.* 81(1):145–66

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, et al. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell*. 129(7):1311–23

Robinson MO, McCarrey JR, Simon MI. 1989. Transcriptional regulatory regions of testis-specific PGK2 defined in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 86(21):8437–41

Romano G, Veneziano D, Acunzo M, Croce CM. 2017. Small non-coding RNA and cancer

Sanyal A, Lajoie BR, Jain G, Dekker J. 2012. The long-range interaction landscape of gene promoters. *Nature*. 489(7414):109–13

Schäfer M, Nayernia K, Engel W, Schäfer U. 1995. Translational control in spermatogenesis. *Dev. Biol.* 172(2):344–52

Shima JE, McLean DJ, McCarrey JR, Griswold MD. 2004. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biol. Reprod.* 71(1):319–30

Shirakata Y, Hiradate Y, Inoue H, Sato E, Tanemura K. 2014. Histone h4 modification during mouse spermatogenesis. *J. Reprod. Dev.* 60(5):383–87

Simpson AJG, Caballero OL, Jungbluth A, Chen Y-T, Old LJ. 2005. Cancer/testis antigens, gametogenesis and cancer. *Nat. Rev. Cancer*. 5(8):615–25

Sinha Hikim AP, Swerdloff RS. 1999. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod*. 4(1):38–47

Sproul D, Gilbert N, Bickmore WA. 2005. The role of chromatin structure in regulating the expression of clustered genes. *Nat. Rev. Genet.* 6(10):775–81

Standaert L, Adriaens C, Radaelli E, Van Keymeulen A, Blanpain C, et al. 2014. The long noncoding RNA Neat1 is required for mammary gland development and lactation. *RNA*. 20(12):1844–49

Su Y, Liebhaber S a, Cooke NE. 2000. The human growth hormone gene cluster locus control region supports position-independent pituitary- and placenta-specific expression in the transgenic mouse. *J. Biol. Chem.* 275(11):7902–9

Sun J, Lin Y, Wu J. 2013. Long non-coding RNA expression profiling of mouse testis during postnatal development. *PLoS One*. 8(10):e75750

Szutorisz H, Canzonetta C, Georgiou A, Chow C-M, Tora L, Dillon N. 2005. Formation of an active tissue-specific chromatin domain initiated by epigenetic marking at the embryonic stem cell stage. *Mol. Cell. Biol.* 25(5):1804–20

Takano N, Kimura A, Takahashi T. 2009. Two distinct localization patterns of testis-specific serine protease 1 (TESSP1) in the Seminiferous Tubules of the Mouse Testis. *Zoolog. Sci.* 26(4):294–300

Takano N, Matsui H, Takahashi T. 2005. TESSP-1: A novel serine protease gene expressed in the spermatogonia and spermatocytes of adult mouse testes. *Mol. Reprod. Dev.* 70(1):1–10

Tesarik J, Greco E, Mendoza C. 2001. Assisted reproduction with in-vitro-cultured testicular spermatozoa in cases of severe germ cell apoptosis: A pilot study. *Hum. Reprod.* 16(12):2640-45

Todeschini A-L, Georges A, Veitia RA. 2014. Transcription factors: specific DNA binding and specific gene regulation. *Trends Genet.* 30(6):211–19

Tolhuis B, Palstra RJ, Splinter E, Grosveld F, de Laat W. 2002. Looping and interaction between hypersensitive sites in the active beta-globin locus. *Mol. Cell*. 10(6):1453–65

Tran N, Su H, Khodadadi - Jamayran A, Lin S, Zhang L, et al. 2016. The AS - RBM15 lncRNA enhances RBM15 protein translation during megakaryocyte differentiation. *EMBO Rep.* 17(6):887–900

Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, et al. 2010. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol. Cell*. 39(6):925–38

Tsai YC, Cooke NE, Liebhaber SA. 2016. Long-range looping of a locus control region drives tissue-specific chromatin packing within a multigene cluster. *Nucleic Acids Res.* 44(10):4651–64

Ulitsky I, Shkumatava A, Jan CH, Sive H, Bartel DP. 2011. Conserved function of lincRNAs in vertebrate embryonic development despite rapid sequence evolution. *Cell*. 147(7):1537–50

Umlauf D, Fraser P, Nagano T. 2008. The role of long non-coding RNAs in chromatin structure and gene regulation: variations on a theme. *Biol. Chem.* 389(4):323–31

van der Heyden MAG, Defize LHK. 2003. Twenty one years of P19 cells: what an embryonal carcinoma cell line taught us about cardiomyocyte differentiation. *Cardiovasc. Res.* 58(2):292–302

Vourekas A, Alexiou P, Vrettos N, Maragkakis M, Mourelatos Z. 2016. Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm. *Nature*. 531(7594):390–94

Wang J, Zhang J, Zheng H, Li J, Liu D, et al. 2004. Mouse transcriptome: Neutral evolution of "non-coding" complementary DNAs. *Nature*. 431(7010):

Wang KC, Chang HY. 2011. Molecular mechanisms of long noncoding RNAs. *Mol. Cell*. 43(6):904–

14

Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, et al. 2011. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. *Nature*. 472(7341):120–24

Wang PJ, Page DC, McCarrey JR. 2005. Differential expression of sex-linked and autosomal germ-cell-specific genes during spermatogenesis in the mouse. *Hum. Mol. Genet.* 14(19):2911–18

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

Whyte WA, Orlando DA, Hnisz D, Abraham BJ, Lin CY, et al. 2013. Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell*. 153(2):307–19

Wichman L, Somasundaram S, Breindel C, Valerio DM, McCarrey JR, et al. 2017. Dynamic expression of long noncoding RNAs reveals their potential roles in spermatogenesis and fertility. *Biol. Reprod.* 97(2):313–23

Wilusz JE, Sunwoo H, Spector DL. 2009. Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev.* 23(13):1494–1504

Wong GW, Yasuda S, Morokawa N, Li L, Stevens RL. 2004. Mouse Chromosome 17A3.3 Contains 13 Genes That Encode Functional Tryptic-like Serine Proteases with Distinct Tissue and Cell Expression Patterns. *J. Biol. Chem.* 279(4):2438–52

Wu YY, Yang Y, Xu Y De, Yu HL. 2015. Targeted disruption of the spermatid-specific gene Spata31 causes male infertility. *Mol. Reprod. Dev.* 82(6):432–40

Xiang J-F, Yin Q-F, Chen T, Zhang Y, Zhang X-O, et al. 2014. Human colorectal cancer-specific CCAT1-L lncRNA regulates long-range chromatin interactions at the MYC locus. *Cell Res.* 24(5):513–31

Xu H, Shen L, Chen X, Ding Y, He J, et al. 2016. mTOR/P70S6K promotes spermatogonia proliferation and spermatogenesis in Sprague Dawley rats. *Reprod. Biomed. Online*. 32(2):207–17

Xu J, Sankaran VG, Ni M, Menne TF, Puram R V., et al. 2010. Transcriptional silencing of γ-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes Dev.* 24(8):783–89

Yadav RP, Kotaja N. 2014. Small RNAs in spermatogenesis. *Mol. Cell. Endocrinol.* 382(1):498–508

Yan W, Si Y, Slaymaker S, Li J, Zheng H, et al. 2010. Zmynd15 encodes a histone deacetylase-dependent transcriptional repressor essential for spermiogenesis and male fertility. *J. Biol. Chem.* 285(41):31418–26

Yang F, Xue X, Zheng L, Bi J, Zhou Y, et al. 2014. Long non-coding RNA GHET1 promotes gastric carcinoma cell proliferation by increasing c-Myc mRNA stability. *FEBS J.* 281(3):802–13

Yang L, Lin C, Jin C, Yang JC, Tanasa B, et al. 2013a. LncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. *Nature*. 500(7464):598–602

Yoneda R. 2014. *Studies on the function and regulation of testis-specific serine proteases during murine spermatogenesis*. PhD Thesis of Hokkaido University

Yoneda R, Kimura AP. 2013. A testis-specific serine protease, Prss41/Tessp-1, is necessary for the progression of meiosis during murine in vitro spermatogenesis. *Biochem. Biophys. Res. Commun.* 441(1):120–25

Yoneda R, Takahashi T, Matsui H, Takano N, Hasebe Y, et al. 2013. Three testis-specific paralogous serine proteases play different roles in murine spermatogenesis and are involved in germ cell survival during meiosis. *Biol. Reprod.* 88(5):1–14

Yu W, Gius D, Onyango P, Muldoon-Jacobs K, Karp J, et al. 2008. Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA. *Nature*. 451(7175):202–6

Zambrowicz BP, Harendza CJ, Zimmermann JW, Brinster RL, Palmiter RD. 1993. Analysis of the mouse protamine 1 promoter in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 90(11):5071–75

Zaratiegui M, Irvine D V, Martienssen RA. 2007. Noncoding RNAs and gene silencing. *Cell*. 128(4):763–76

Zhang E, Kong R, Yin D, You L, Sun M, et al. 2014. Long noncoding RNA ANRIL indicates a poor

prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/miR-449a. *Oncotarget*. 5(8):2276–92

Zhu Y, Rowley MJ, Böhmdorfer G, Wierzbicki AT. 2013a. A SWI/SNF Chromatin-Remodeling Complex Acts in Noncoding RNA-Mediated Transcriptional Silencing. *Mol. Cell*. 49(2):298–309

Zhu Y, Sun L, Chen Z, Whitaker JW, Wang T, Wang W. 2013b. Predicting enhancer transcription and activity from chromatin modifications. *Nucleic Acids Res.* 41(22):10032–43

Zirkin BR, Chen H. 2000. Regulation of Leydig cell steroidogenic function during aging. *Biol. Reprod.* 63:977–81

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TABLES and FIGURES

Table 1. Stage-specific genes in spermatogenesis

gene name	expression	function	Reference
TPAP	predominantly in round spermatids	government of germ cell morphogenesis by modulating specific transcription factors inhibitition of germ cell apoptosis	Kashiwabara, 2002
Brdt	nuclei of round spermatids	remodeling of the chromatin architecture in sperm	Berkovits and Wolgemuth, 2011
H3t	differentiating spermatogonia to spermatocytes	entry into spermatogenesis spermatogonial differentiation	Ueda et al., 2017
SHIP1	nuclei of spermatocytes and round spermatids	involvement in chromatin reorganization of spermatids	Rathke et al., 2014
Zmynd15	predominantly in pachytene spermatocytes and spermatids	inhibition of germ cell apoptosis in spermatocytes and spermatids	Yan et al., 2010
H2A.L.2	elongating spermatids	contribution to sperm genome compaction	Barral et al., 2017
Hsp70-2	highly expressed in pachytene spermatocytes	inhibition of germ cell apoptosis in spermatocyes	Dix et al., 1996
Spata31	spermatids	inhibition of the lack of spermatid inhibition of appearance of abnomal phenotype of germ cells	Wu et al., 2015
Spaca1	spermatids	promotion of formation of intermediate filament bundles on the nuclear membrane	Fujihara et al., 2012
Kit	spermatogonia	a crucial impact on germ cell survival and thus male fertility	Correina et al., 2014
Nkapl	pachytene spermatocytes to spermatids	germ cell maturation and inhibit apoptosis in pachytene spermatocytes	Okuda et al., 2015

Table 2. Primers used in chapter 1

Digestion	forward	reverse
DNase I HS mapping		
Probe for BamHI and HindIII fragments	5′-GGCAATCTCTGATCTTACCC-3'	5'-ACAGCAAGGAACTCCTGACA-3'
Probe for EcoRI fragment	5′-TCAGGCACATGCATGTCTGT-3'	5′-CCAAGGAGTCTGCTTCTCTT-3'
Reverse-transcription PCR 5′-TCC TCC TTC TGT CCC TCA GA		
lncRNA-HSVIII	5′-CACTGTTCCTTGACTTGAT-3'	5'-GTGTGTATTCAGGTATGTGC-3'
Gapdh	5′-CATGACCACAGTCCATGCCATC-3'	5′-TAGCCCAAGATGCCCTTCAGTG-3'
Gapdh (in5-ex6)	5'-CCTTCTTTGTAGGTGTCCCT-3'	5'-TAGCCCAAGATGCCCTTCAGTG-3'
Gapdh (ex5-ex6)	5′-TTGTGATGGGTGTGAACCAC-3'	5′-TAGCCCAAGATGCCCTTCAGTG-3'
5'RACE and 3'RACE		
Abridged anchor primer	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'	
Abridged universal amplification primer	5′-GGCCACGCGTCGACTAGTAC-3'	
3 sites adapter primer		5′-CTGATCTAGAGGTACCGGATCC-3'
RT-1		5'-TGCAGCTTGAAAAATGTGAA-3'
GSP1		5′-CACTACCGAATCACAAGATC-3'
GSP2		5′-ATCAAGTCAAGGAACAGGTG-3'
GSP3	5′-AGTATAGAGGAGCTCTTATC-3'	
GSP4	5′-GTGCATGAGGGCCTTAGTTT-3'	
3C assay		
Anchor primer	5′-CATAAGGCGTAGAAAATACG-3'	
HSI		5′-TGGAGAGAGGGAAAGAAGGA-3'
HSIV		5′-TTGTCTTTAGTATGGAGTGT-3'
Tessp-2 promoter		5′-CCTCCATCTCCCAGTAAGGT-3’
Tessp-3 promoter		5′-TCCTCCTTCTGTCCCTCAGA-3'
Tessp-4 promoter		5′-CCACTGCCTGGCAAGAATTT-3'
Tessp-4 3′ region		5′-CCAGGAGGTTGTGTAGCAAA-3'
Tessp-3 and *Tessp-4* intergenic region		5′-GGCTTCTCAAGATTATAGTG-3'
Ercc3 forward-1	5′-CTCTTCCTGGGTCTGTCGAT-3'	
Ercc3 forward-2	5′-TGAGATCGTCACACGTCCTT-3'	
Plasmid constructs		
Tessp-2 promoter	5′-CCAAGTACACTGTAGCTGTC-3'	5′-GTCATCACGTAGGCCACCCT-3'
5' half of *lncRNA-HSVIII*	5′-TTCATCTTCCCTCACTAGTCATCATTTG-3'	5′-ACCAGGCTGGCTTCGAACTTAC-3'
3' half of *lncRNA-HSVIII*	5′-AGTATAGAGGAGCTCTTATC-3'	5′-TTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTCTCTGTGT-3'

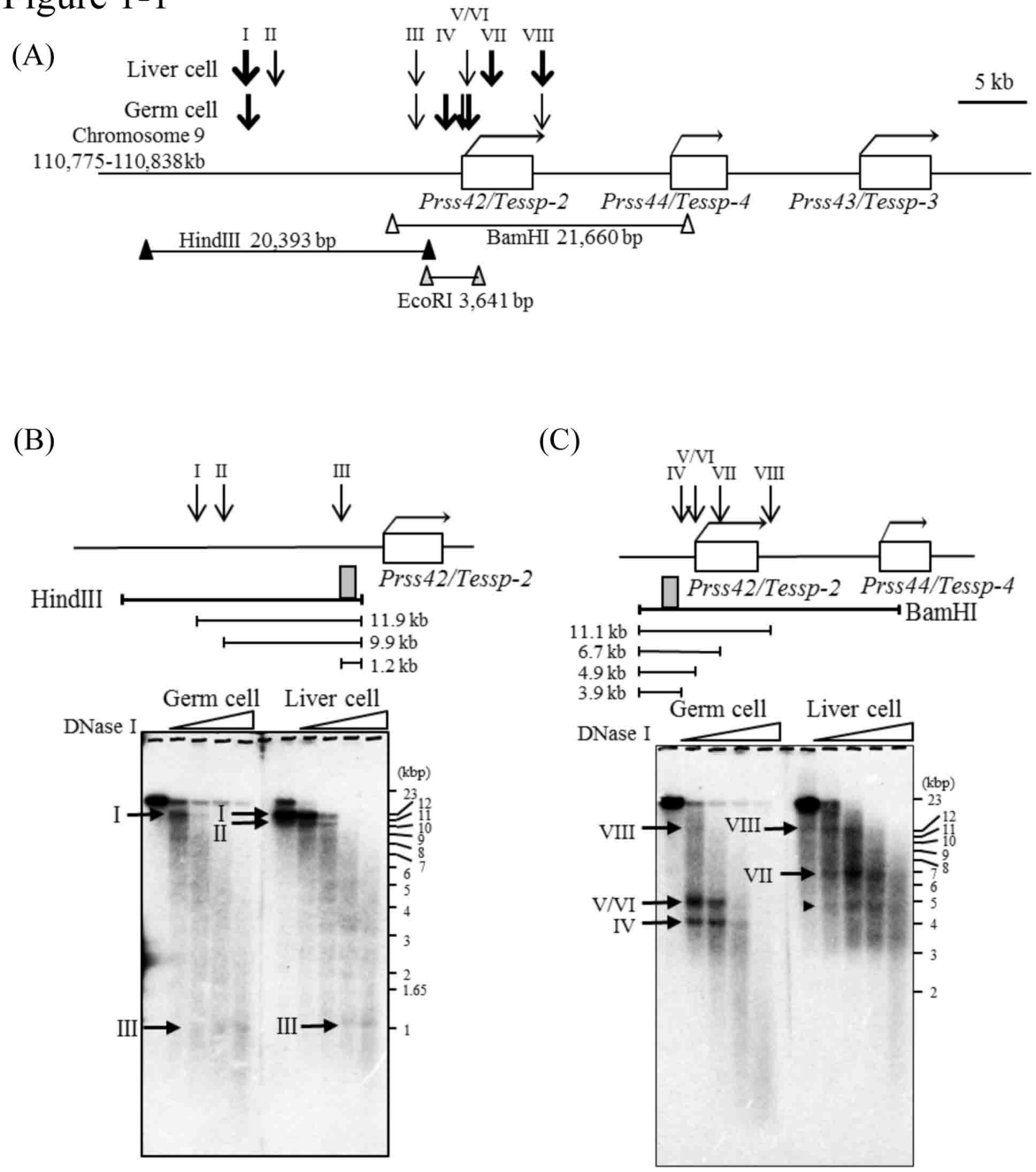
Table 3. Primers used in chapter 2

Designation	forward	Reverse
RT-PCR		
Tesra PCR	5'-CGTGCCTGTTTCTTTCCATT-3'	5'-CAGCCACCATGTTCTGTGAC-3'
Tesra 2890bp	5'-CGTGCCTGTTTCTTTCCATT-3'	5'-GTTGCATCTCCACCCATCA-3'
Tesra full length	5'-AGAGGTGTGAGGCTGGTGGG-3'	5'-TGAACATATTAAATGAGCTC-3'
5' and 3' RACE		
GSP1		5'-CAGCCACCATGTTCTGTGAC-3'
GSP2		5'-GGCAACCTGGAAAGGAAAAT-3'
GSP3		5'-AATGGAAAGAAACAGGCACG-3'
GSP4	5'-CCTGAAGCTCACCCCTAGTA-3'	
GSP5	5'-TTGATGGGTGGAGATGCAAC-3'	
ChIRP PCR		
Rec8 promoter	5'-CCTTGGCGAAGTCCTTGTTA-3'	5'-AATAGTCGCCAGCCAATCAC-3'
B2m promoter	5'-GGCCAGGGGTTTAACTTCTC-3'	5'-CCCTTGGGGTTTCTGCTTAT-3'
Tessp-2 promoter	5'-GGTGCCTCTTTGGAGACCTA-3'	5'-GACCCCTTACTTCCTGTGGA-3'
Tessp-4 promoter	5'-CTTCTGCTGTCTGGGAGTCA-3'	5'-AACGGGTGGTTTCATTTAGC-3'
Tessp-3 promoter	5'-CTGCAGGTGACTCCACACTT-3'	5'-TTCCTCCTTCTGTCCCTCAG-3'
intergenic *Tessp*2-4	5'-CAGAGTCGCCTTCAAAATCC-3'	5'-TAGCCCAAATCAGCACCTTC-3'
intergenic *Tessp*4-3	5'-GAGGTACAGGCACAAGCACA-3'	5'-TAGCTGTGGCAGGCTTAGGT-3'
Prss45 promoter	5'-AGCACTAGGGGTGCTGTCAT-3'	5'-TGGACCCACTCTCGTCCTTA-3'
Prss46 promoter	5'-TCAGTGGAGACTGGCATGAA-3'	5'-CCTGTGACCTTGTCCCTTGT-3'
Prss50 promoter	5'-GCAGAGGAGGGTAGGGGTAT-3'	5'-TATGCCTCGCCTCAGCTAAT-3'

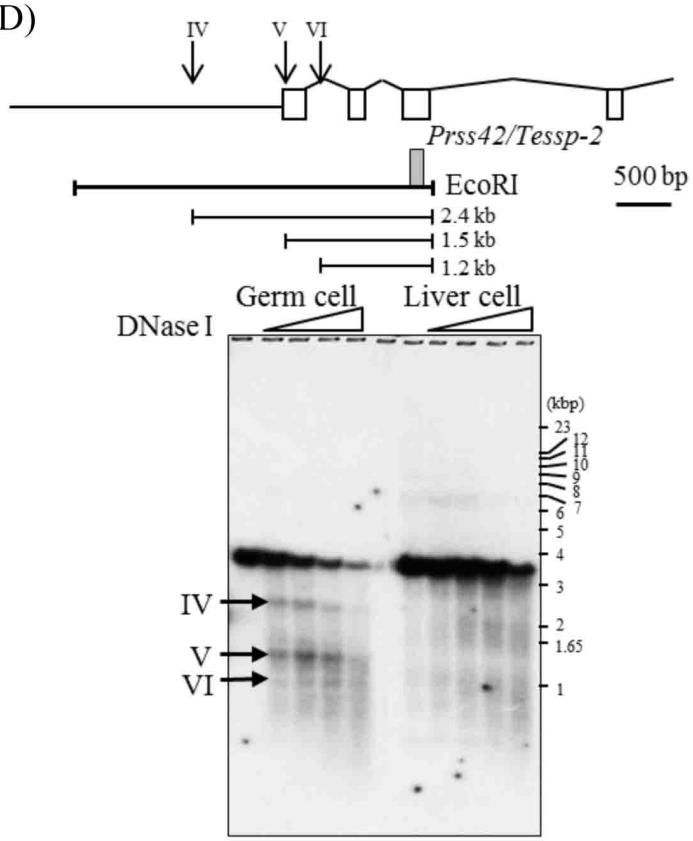
Table 4. Primers used for ChIRP assay

Designation	forward	Reverse
ChIRP tiling oligo		
ChIRP-1		5'-CTAACATCTATCCTCTCCAA-3'
ChIRP-2		5'-GCCTCTAAAGTCAGGAGAAC-3'
ChIRP-3		5'-TCAAGGCAACCTGGAAAGGA-3'
ChIRP-4		5'-TACCAGTGCTTGACTTAGTA-3'
ChIRP-5		5'-GGCATTTCTTACTTCACACA-3'
ChIRP-6		5'-CCAGTCTCTTAGGGATTTAT-3'
ChIRP-7		5'-TACCTAACACTTCTTGCACT-3'
ChIRP-8		5'-ACTGGAGCAGCAAGAATTGC-3'
ChIRP-9		5'-GCTAGGCAAACTTCACCAAG-3'
ChIRP-10		5'-TCCACCCATCAAGACATTAA-3'
ChIRP-11		5'-AGAGAATTGGTGCCATGTTC-3'
ChIRP-12		5'-CTACTGCTCCGATGAAATGT-3'
ChIRP-13		5'-CAGTTTATTATCTGCTCACC-3'

Figure 1-1


(A)
Liver cell
Germ cell
Chromosome 9
110,775-110,838kb
I II
III IV V/VI VII VIII
5 kb
Prss42/Tessp-2
Prss44/Tessp-4
Prss43/Tessp-3
HindIII 20,393 bp
BamHI 21,660 bp
EcoRI 3,641 bp
(B)
I II III
Prss42/Tessp-2
HindIII
11.9 kb
9.9 kb
1.2 kb
Germ cell
Liver cell
DNase I
(kbp)
I
II
III
(C)
IV V/VI VII VIII
Prss42/Tessp-2
Prss44/Tessp-4
BamHI
11.1 kb
6.7 kb
4.9 kb
3.9 kb
Germ cell
Liver cell
DNase I
(kbp)
VIII
VII
V/VI
IV

(D)


IV
V
VI
Prss42/Tessp-2
EcoRI
500 bp
2.4 kb
1.5 kb
1.2 kb
Germ cell
Liver cell
DNase I
(kbp)
23
12
11
10
9
8
7
6
5
4
3
2
1.65
1
IV
V
VI

(E)

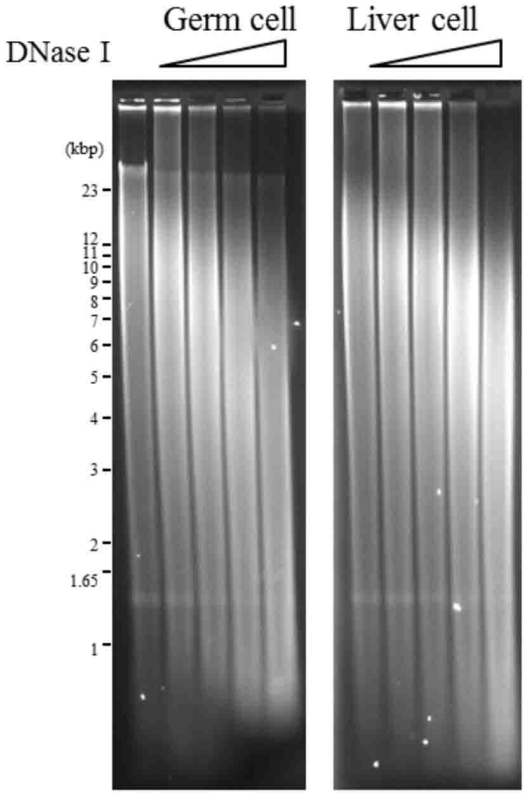
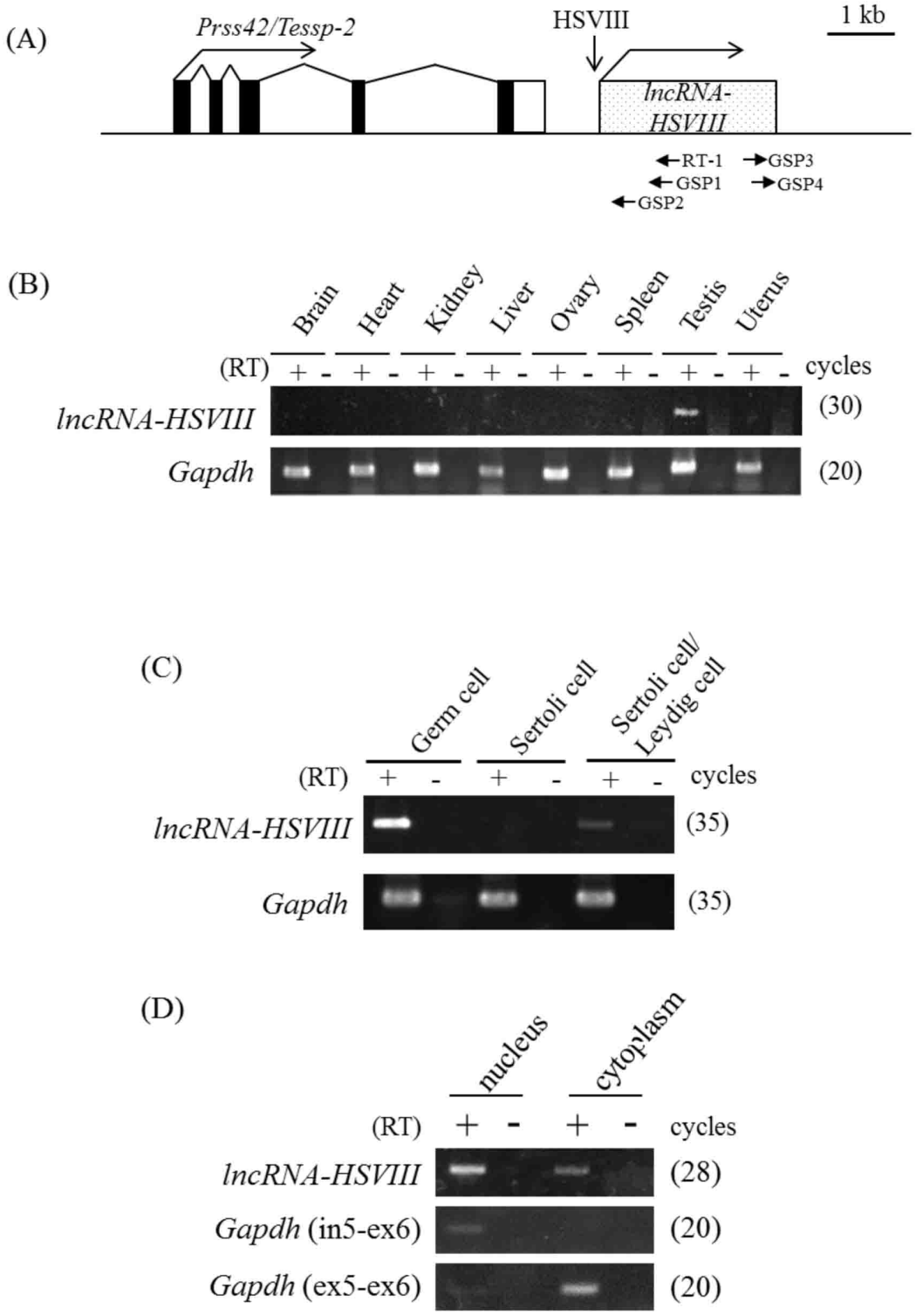

Germ cell
Liver cell
DNase I
(kbp)
23
12
11
10
9
8
7
6
5
4
3
2
1.65
1

Figure 1-1. DNase I HS mapping at the mouse *Prss/Tessp* locus. A: Schematic drawing of the *Prss/Tessp* gene cluster showing the position of identified DNase I HSs in germ cells and liver cells by vertical arrows. A 63-kb region corresponding to 110,775-110,838 kb of mouse chromosome 9 is drawn. The width of the arrow reflects the signal intensity of each DNase I HS. Three cluster genes are depicted with white boxes, and bent arrows indicate the direction of transcription. A ~38-kb sequence encompassing the *Prss42/Tessp-2* gene was searched by digestion with *Bam*HI, *Hin*dIII, or *Eco*RI. Restriction sites and fragment sizes are shown below the gene structure. **B,C:** Southern blot detecting DNase I HSs on the *Hin*dIII (B) or *Bam*HI (C) fragment. Nuclei from native testicular germ cells and liver cells were treated with DNase I for 0, 15, 45, 90, or 180 sec, and then genomic DNA was purified. After restriction digestion, DNAs were transferred to a nylon membrane and hybridized with a radiolabeled probe (gray box). Signals were detected by autoradiography. The *Prss42/Tessp-2* gene and its upstream region are drawn above each blot, with each restriction fragment as well as the fragments detected as a result of DNase I treatment. Sizes of DNase I-digested fragments are shown, and positions of DNase I HSs are indicated with their numbers by vertical arrows. Image of the Southern blot are presented below each diagram. Bands corresponding to DNase I HSs are indicated with horizontal arrows. Molecular size markers are shown at the right. An arrowhead in ‘‘C’’ shows the signal that was not detected by *Eco*RI digestion in liver cells. **D:** Fine mapping of the *Prss42/Tessp-2* promoter region. DNase I treatment of nuclei and restriction digestion with *Eco*RI followed by Southern blot were performed as in ‘‘B’’. A promoter region and the first four exons of the *Prss42/ Tessp-2* gene are drawn above the blot. Positions of DNase-I HSs are indicated by vertical arrows, and exons are depicted with white boxes connected by bent lines. An *Eco*RI fragment is shown below the gene structure, with the fragments detected after DNase I treatment and the probe for Southern blot indicated by a gray box. The Southern blot is presented as in ‘‘B’’. **E:** Ethidium

bromide staining of the gels used in “D”. Nuclei from testicular germ cells and liver cells were treated with DNase I for 0-180 sec at 37^{O}C, and the purified DNAs were digested with *Eco*RI. The DNAs were then separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Positions of molecular size marker bands are shown at left. The smear band gets shorter as the time of the DNase I-treatment gets longer.

Figure 1-2


(A)
Prss42/Tessp-2
HSVIII
1 kb
lncRNA-HSVIII
RT-1
GSP3
GSP1
GSP4
GSP2
(B)
Brain
Heart
Kidney
Liver
Ovary
Spleen
Testis
Uterus
(RT) + - + - + - + - + - + - + - + - cycles
lncRNA-HSVIII
(30)
Gapdh
(20)
(C)
Germ cell
Sertoli cell
Sertoli cell/ Leydig cell
(RT) + - + - + - cycles
lncRNA-HSVIII
(35)
Gapdh
(35)
(D)
nucleus
cytoplasm
(RT) + - + - cycles
lncRNA-HSVIII
(28)
Gapdh (in5-ex6)
(20)
Gapdh (ex5-ex6)
(20)

(E)

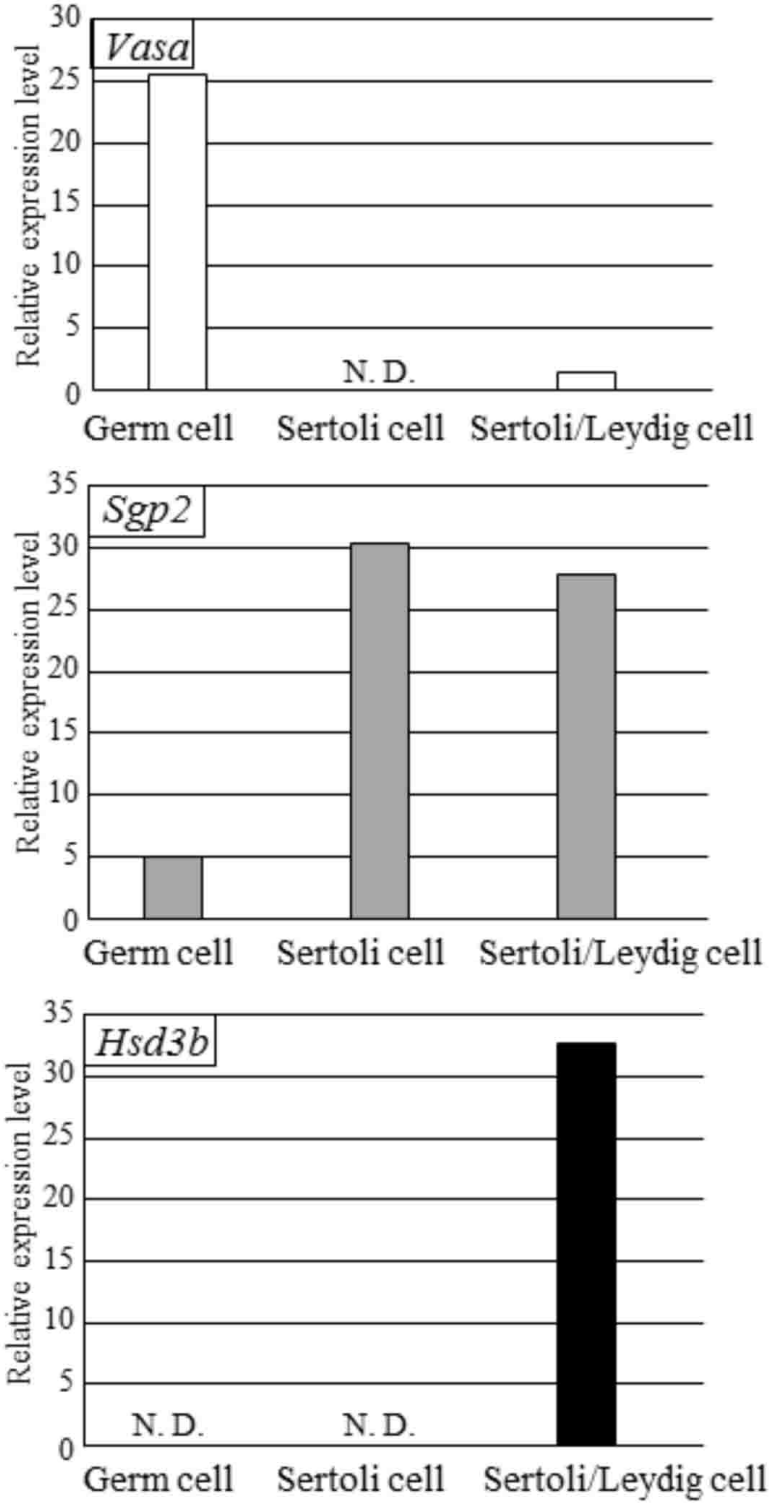

Vasa
Relative expression level
30
25
20
15
10
5
0
N. D.
Germ cell
Sertoli cell
Sertoli/Leydig cell
Sgp2
Relative expression level
35
30
25
20
15
10
5
0
Germ cell
Sertoli cell
Sertoli/Leydig cell
Hsd3b
Relative expression level
35
30
25
20
15
10
5
0
N. D.
N. D.
Germ cell
Sertoli cell
Sertoli/Leydig cell

Figure 1-2. Characterization and expression of *lncRNA-HSVIII*. A: Schematic drawing of the *Prss42/Tessp-2* gene and *lncRNA-HSVIII*. Exons of the *Prss42/Tessp-2* gene are depicted as coding regions (black boxes) and an untranslated region (white box). *lncRNA-HSVIII* contains no intron. Bent arrows indicate the transcriptional direction of the *Prss42/Tessp-2* gene and *lncRNA-HSVIII*. A vertical arrow shows the position of DNase I HSVIII. Positions of the primers used in 5'RACE and 3' RACE are indicated by short horizontal arrows, located below the gene structure. **B:** Tissue specificity of *lncRNA-HSVIII* was examined by reverse-transcription PCR. Total RNA was isolated from eight mouse tissues, as indicated, and reverse transcription was performed using the oligo(dT) primer with (RT+) or without reverse transcriptase (RT-). The cycle number of each PCR reaction is indicated in the parentheses at right. *Gapdh* abundance was examined as an internal control. *lncRNA-HSVIII* was specifically expressed in the testis. **C:** Localization of *lncRNA-HSVIII* in the mouse testis was examined by reverse-transcription PCR using cDNAs obtained from fractionated, native testicular cells. Germ-cell, Sertoli-cell, and Sertoli/Leydig-cell fractions were isolated from adult mouse testes, while Sertoli cells were collected by primary culture from immature testes. The expression of marker genes for these fractions was evaluated by quantitative reverse-transcription PCR as shown in "E". Reverse-transcription PCR was performed as in ''B'', and the cycle number of each PCR is indicated in the parentheses at right. *lncRNA-HSVIII* was mainly expressed in germ cells, and to a lower degree in Leydig cells. **D:** Subcellular localization of *lncRNA-HSVIII*. Native testicular germ cells were fractionated into the nucleus and cytoplasm, and reverse-transcription PCR was performed as in ''B''. Two sets of primers were used to check the quality of the two fractions. *Gapdh* (in5-ex6) was amplified to detect immature mRNA in the nucleus, and *Gapdh* (ex5-ex6) was used to identify mature mRNA in the cytoplasm. These two PCR assessments indicate that our subcellular fractionation was successful. The *lncRNA-HSVIII* signal was detected in both fractions, but the nuclear signal was stronger. **E:** Expression of marker genes in testicular cell fractions. The expression

of marker genes was examined by qRT-PCR with cDNAs obtained from fractionated germ cells, Sertoli cells, and Sertoli/Leydig cells. *Vasa*, *Sgp2*, and *Hsd3b* were used as markers for germ cells, Sertoli cells and Leydig cells, respectively. The expression level of each marker gene was normalized to that of *Aip*. N. D.; not detected.

Figure 1-3

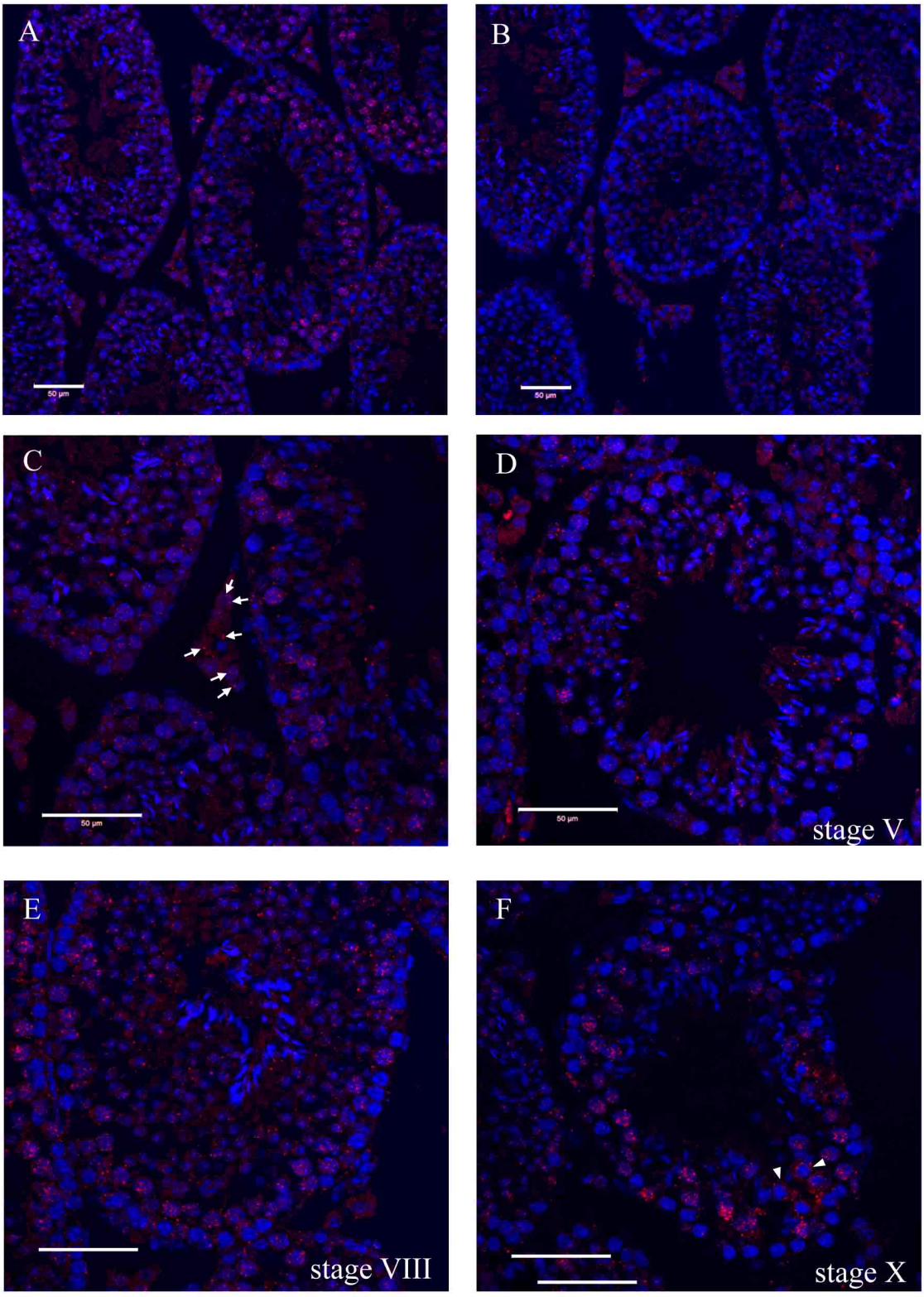


Figure 1-3. Localization of *lncRNA-HSVIII* in the mouse testis. In situ hybridization analysis of *lncRNA-HSVIII* in the adult mouse testis was performed with the tyramide signal amplification system. The testis was fixed with paraformaldehyde, embedded in paraffin, and cut into 7-μm sections. The sections were hybridized with digoxigenin-labeled sense or antisense probe for *lncRNA-HSVIII*, and subsequently incubated with anti-digoxigenin-HRP antibody. Positive signal was detected by the reaction with tyramide-Cy3 (red dots), and nuclei were stained with Hoechst33258 (blue). **A:** Testis section hybridized with the antisense probe of *lncRNA-HSVIII*. Positive signals (red dots) were observed in all seminiferous tubules and in interstitial Leydig cells. **B:** Testis section hybridized with the sense probe of *lncRNA-HSVIII*; few red dots were seen. **C:** An enlarged image of Leydig cells on the section hybridized with the antisense probe. Positive red dots were present in both nuclei and cytosols of Leydig cells (indicated by arrows). **D-F:** Enlarged images of seminiferous tubules at different stages, as indicated. Positive red signals were observed in nuclei of most pachytene spermatocytes and in the cytosol of some pachytene spermatocytes (arrowheads) and all spermatids. Scale bars, 50 mm.

Figure 1-4

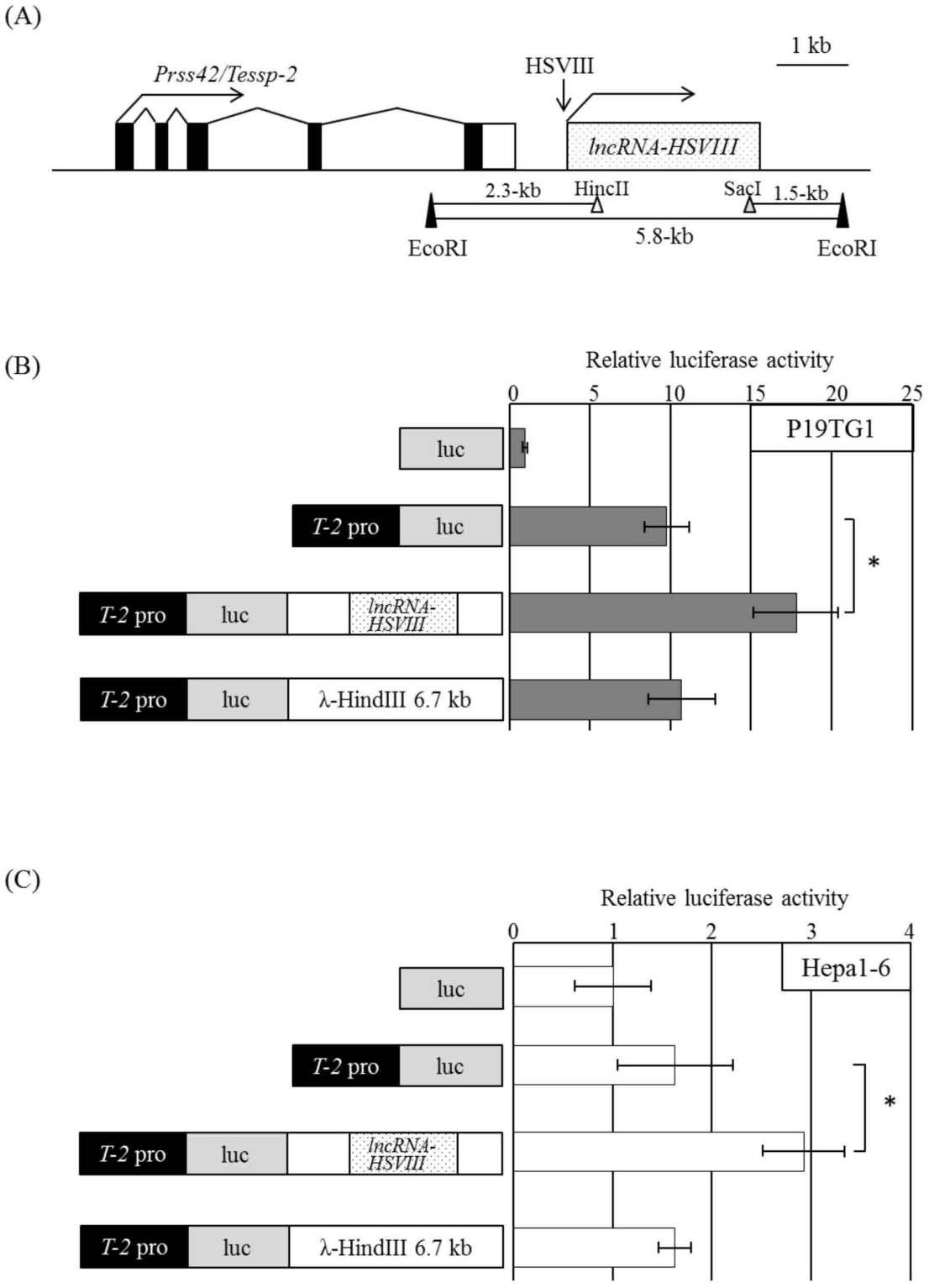

(A)
1 kb
Prss42/Tessp-2
HSVIII
lncRNA-HSVIII
2.3-kb
HincII
SacI
1.5-kb
5.8-kb
EcoRI
EcoRI
(B)
Relative luciferase activity
0
5
10
15
20
25
P19TG1
luc
T-2 pro
luc
*
T-2 pro
luc
lncRNA-HSVIII
T-2 pro
luc
λ-HindIII 6.7 kb
(C)
Relative luciferase activity
0
1
2
3
4
Hepa1-6
luc
T-2 pro
luc
*
T-2 pro
luc
lncRNA-HSVIII
T-2 pro
luc
λ-HindIII 6.7 kb

Figure1-4. Enhancer activity of the 5.8-kb sequence encompassing *lncRNA-HSVIII*. A: Schematic drawing of the *Prss42/Tessp-2* gene and *lncRNA-HSVIII*. The *Prss42/Tessp-2* locus is drawn as in Figure 1-2A. The position of DNase I HSVIII is indicated by a vertical arrow. A 5.8-kb *Eco*RI fragment encompassing *lncRNA-HSVIII* is indicated below the gene structure. *Hinc*II and *Sac*I were used to obtain 5' and 3' flanking sequences to *lncRNA-HSVIII*, respectively; their sizes are shown. **B,C:** Luciferase assay conducted with constructs indicated at the left side of the graph. The indicated constructs were prepared and transfected into embryonal carcinoma P19TG1 cells (B) or hepatic tumor Hepa1-6 cells (C), and luciferase activity was measured 48hr after transfection. The value from the construct without any promoter was set to 1.0 in each experiment. The 5.8-kb sequence significantly increased *Prss42/Tessp-2* promoter activity in both cells. The data are presented as mean ± S.D. from at least three independent experiments. *P<0.05 relative to the construct without the 5.8-kb sequence and to the construct with a 6.7-kb λDNA.

Figure 1-5

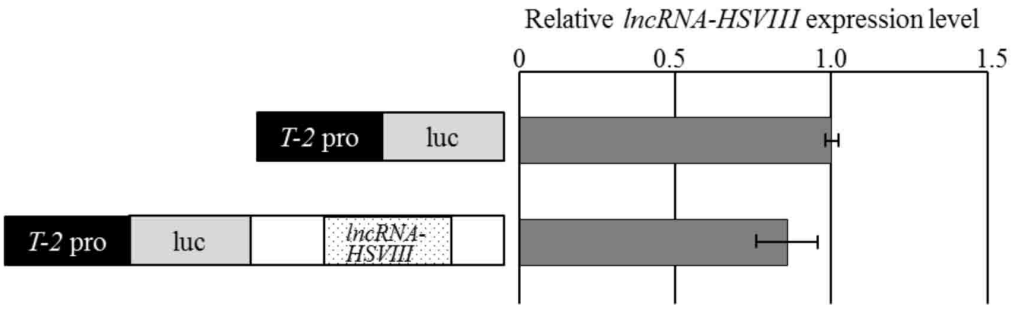


Figure 1-5. No induction of *lncRNA-HSVIII* transcription by the reporter construct. The expression of *lncRNA-HSVIII* was measured by quantitative reverse-transcription PCR with cDNAs obtained from P19TG1 cells that were transfected with the indicated constructs. Transfection of the construct with the 5.8-kb fragment did not result in an increase in *lncRNA-HSVIII* abundance.

Figure 1-6

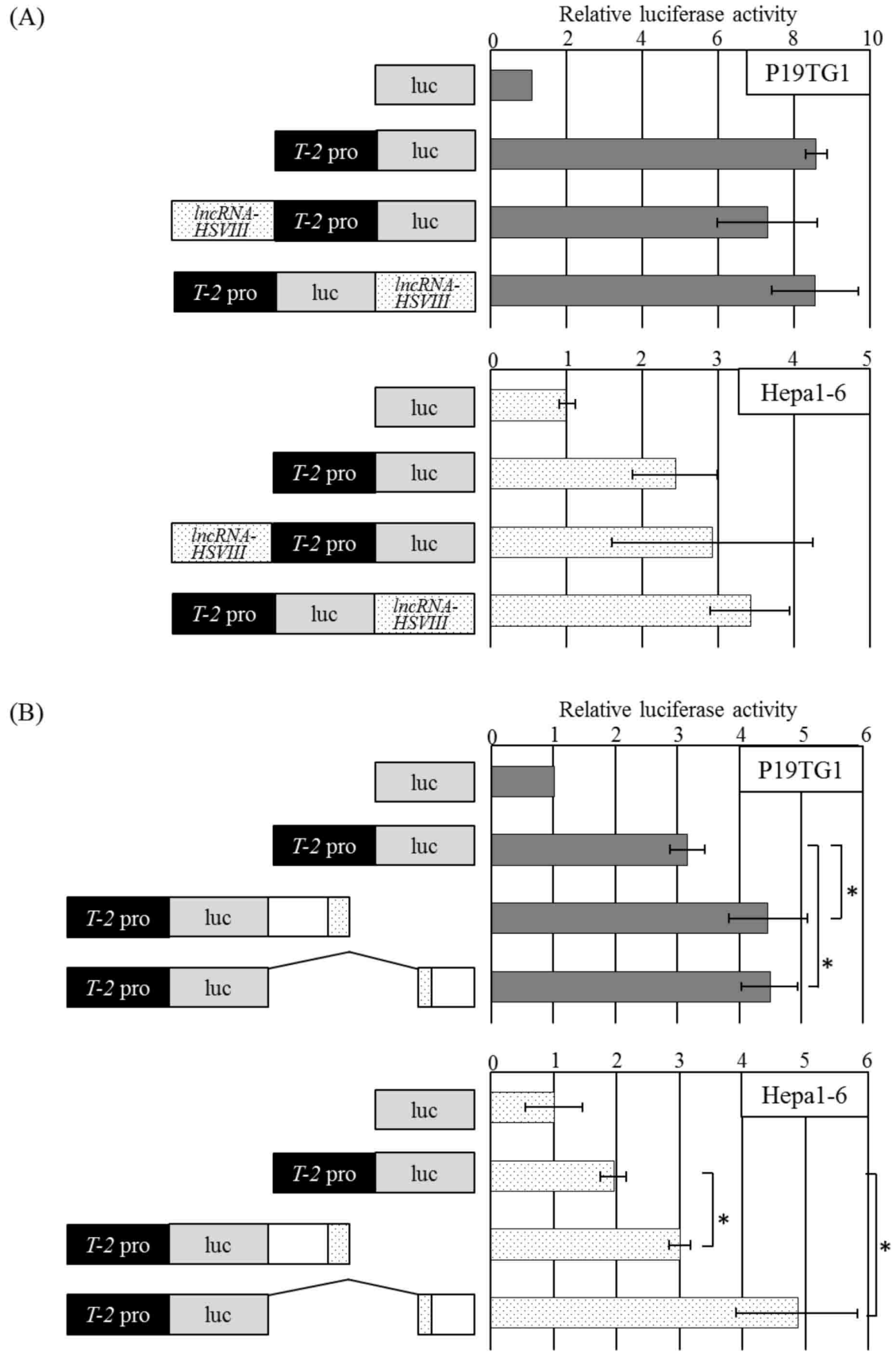

(A)
Relative luciferase activity
P19TG1
Hepa1-6
luc
T-2 pro
lncRNA-HSVIII
(B)
Relative luciferase activity
P19TG1
Hepa1-6
luc
T-2 pro
lncRNA-HSVIII
*

Figure 1-6. **Enhancer activity of sequences flanking *lncRNA-HSVIII*. A:** Enhancer activity of the *lncRNA-HSVIII* sequence was assessed by a reporter-gene assay. Constructs indicated at the left side of the graph were transfected into P19TG1 cells (top) or Hepa1-6 cells (bottom), and luciferase activity was measured 48 hr after transfection. Relative activity was calculated and presented as in Figure 1-4. The *lncRNA-HSVIII* sequence did not possess enhancer activity in either cell type. The data are presented as mean ± S.D. from at least three independent experiments. **B:** Enhancer activity of the upstream and downstream sequence of *lncRNA-HSVIII*. A 2.3-kb upstream and a 1.5-kb downstream sequence of *lncRNA-HSVIII* were obtained by *Hinc*II and *Sac*I restriction digestion of the 5.8-kb fragment, respectively, as indicated in Figure 1-4A. All the constructs were separately transfected into P19TG1 cells and Hepa1-6 cells, and luciferase activity was measured and presented as in Figure 1-4. Both upstream and downstream sequences significantly increased *Prss42/Tessp-2* promoter activity in both cells. The data are presented as mean ± S.D. from at least three independent experiments. *$P < 0.05$ relative to the construct without the 5.8-kb sequence.

Fig. 2-1

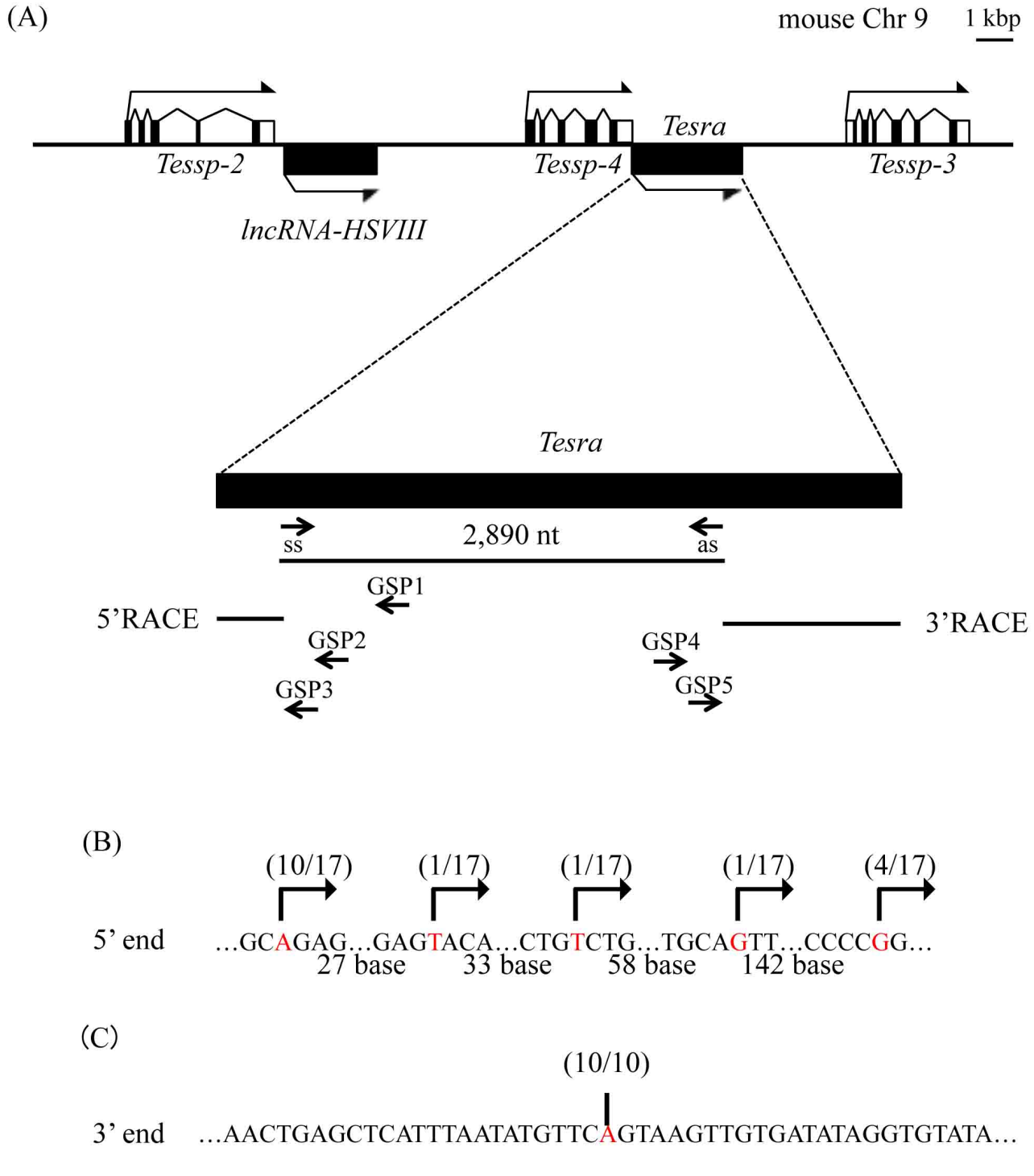

(A)
mouse Chr 9
1 kbp
Tessp-2
Tesra
Tessp-4
Tessp-3
lncRNA-HSVIII
Tesra
ss
2,890 nt
as
GSP1
5'RACE
3'RACE
GSP2
GSP4
GSP3
GSP5
(B)
(10/17)
(1/17)
(1/17)
(1/17)
(4/17)
5' end
…GCAGAG…GAGTACA…CTGTCTG…TGCAGTT…CCCCGG…
27 base
33 base
58 base
142 base
(C)
(10/10)
3' end
…AACTGAGCTCATTTAATATGTTCAGTAAGTTGTGATATAGGTGTATA…

Figure 2-1. Cloning of a novel lncRNA, *Tesra*. A: A schematic view of the *Prss/Tessp* locus on mouse chromosome 9. Exons of three *Prss/Tessp* genes are depicted by black and white boxes representing translated and untranslated regions, respectively. Two lncRNAs that do not contain any introns are also depicted by black boxes. Bent arrows indicate the transcriptional direction. Below the gene structure, the genomic region transcribed into *Tesra* is enlarged. A 2,890-nucleotide sequence was first detected as a transcribed sequence by RT-PCR. 5'RACE was performed with three primers (GSP1-GSP3) and 3'RCAE was with GSP4 and GSP5 primers. The resulting RACE products are indicated by horizontal lines. **B:** 5' end variations of *Tesra* determined by 5'RACE. A sequence around the 5' end of *Tesra* is shown. A specific band was obtained by 5'RACE, and 17 subclones were sequenced. The position of 5' end of each subclone is indicated by bent arrows with the number of subclone. Five TSSs were detected as written by red font, and the most upstream adenine was determined as a main TSS. **C:** 3' end of *Tesra* determined by 3'RACE. A specific band was obtained by 3'RACE, and 10 subclones were checked by DNA sequencing. In all subclones, an adenine was identified as a main TTS, as written by red font and indicated by a vertical line.

Fig. 2-2

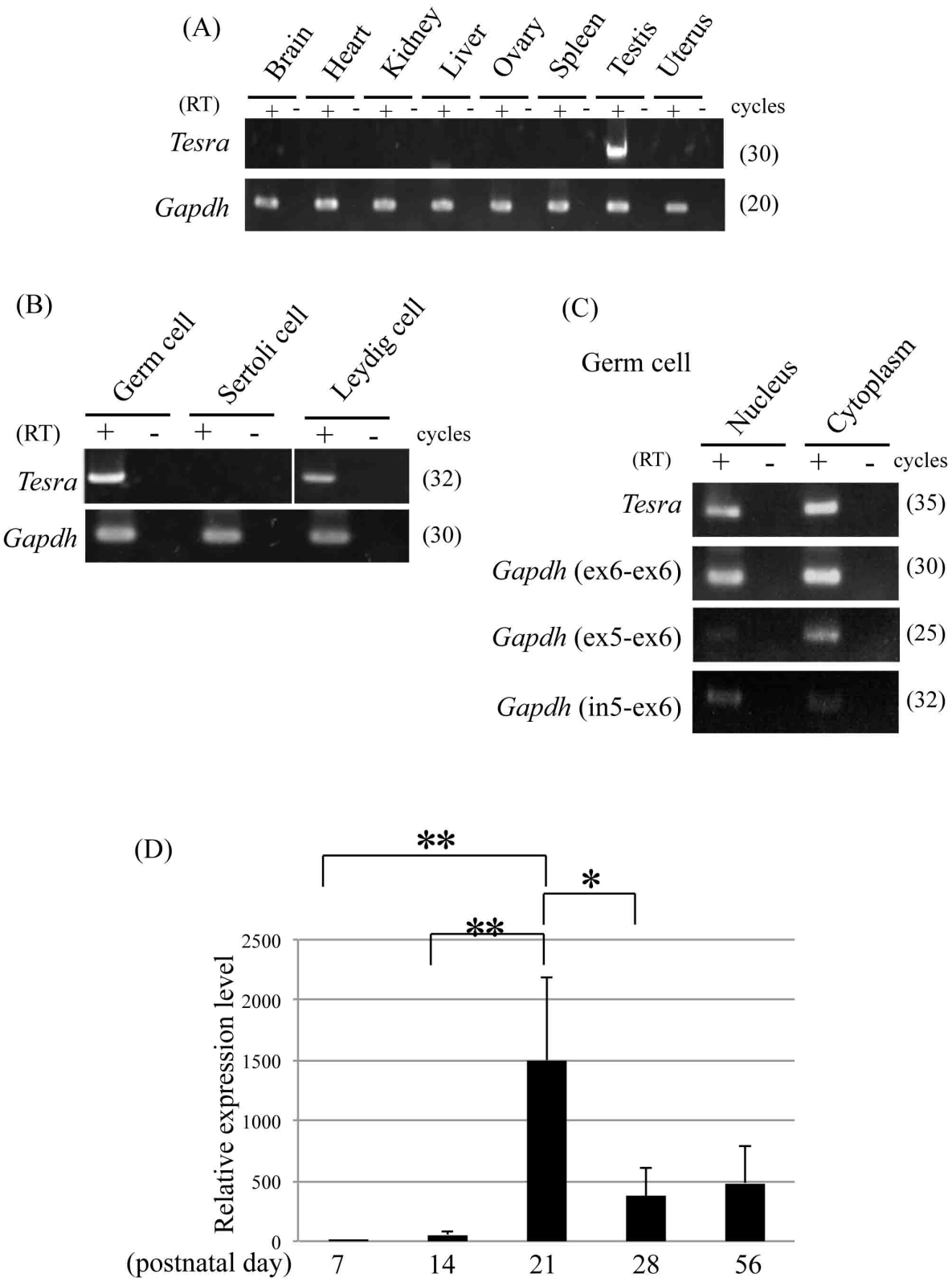

(A)
Brain
Heart
Kidney
Liver
Ovary
Spleen
Testis
Uterus
(RT)
+ - + - + - + - + - + - + - + -
cycles
Tesra
(30)
Gapdh
(20)
(B)
Germ cell
Sertoli cell
Leydig cell
(RT)
+ - + - + -
cycles
Tesra
(32)
Gapdh
(30)
(C)
Germ cell
Nucleus
Cytoplasm
(RT)
+ - + -
cycles
Tesra
(35)
Gapdh (ex6-ex6)
(30)
Gapdh (ex5-ex6)
(25)
Gapdh (in5-ex6)
(32)
(D)
**
*
**
Relative expression level
2500
2000
1500
1000
500
0
(postnatal day)
7
14
21
28
56

Figure 2-2. The expression pattern of *Tesra*. **A:** Tissue specificity of *Tesra*. Eight mouse tissues, as indicated, were collected from adult mice and used for RT-PCR analysis. Reverse transcription was performed with the oligo(dT) primer with (RT+) or without reverse transcriptase (RT-). *Gapdh* was detected as an internal control. The cycle number of each PCR reaction is indicated in parentheses. **B:** Cell type-specificity of *Tesra* in the testis. Mouse germ cell, Sertoli cell, and Leydig cell fractions were prepared and used for RT-PCR analysis. Germ cells and Leydig cells were collected from adult mouse testes. Sertoli cells were collected from 7-12 days old testes and cultured for several days. The experiment was done and the data are presented as in "A". **C:** Subcellular localization of *Tesra* in mouse male germ cells. Germ cells were collected and fractionated into nuclear and cytoplasmic fractions, and total RNAs were purified from both fractions. RT-PCR was done and the data are presented as in "A". *Gapdh* (ex6-ex6) was a positive control, in which both forward and reverse primers were designed within exon 6 of *Gapdh*. By this PCR, the positive signal should appear in both fractions. *Gapdh*(ex5-ex6) was a cytoplasmic marker, because a primer set in exon 5 and exon 6 should only amplify the spliced mRNA. *Gapdh*(in5-ex6) was a marker of nuclear fraction, and could only amplify premature RNA. RT-PCR was done and the data are presented as above. The *Tesra* transcript was detected in both nuclear and cytoplasmic fractions. **D:** *Tesra* expression during postnatal testis development. Mouse testes at postnatal day 7, 14, 21, 28, and 56 were collected, and total RNAs were purified and used for qRT-PCR analysis. The housekeeping *Aip* gene was also examined as an internal control. The level of *Tesra* was normalized to *Aip*, and the value at day 7 was set to 1.0. The data are presented as mean ± S.D. from three independent experiments, and the statistical significance was analyzed by one-way ANOVA followed by Tukey's post hoc test. $*P < 0.05$. $**P < 0.01$.

Fig. 2-3

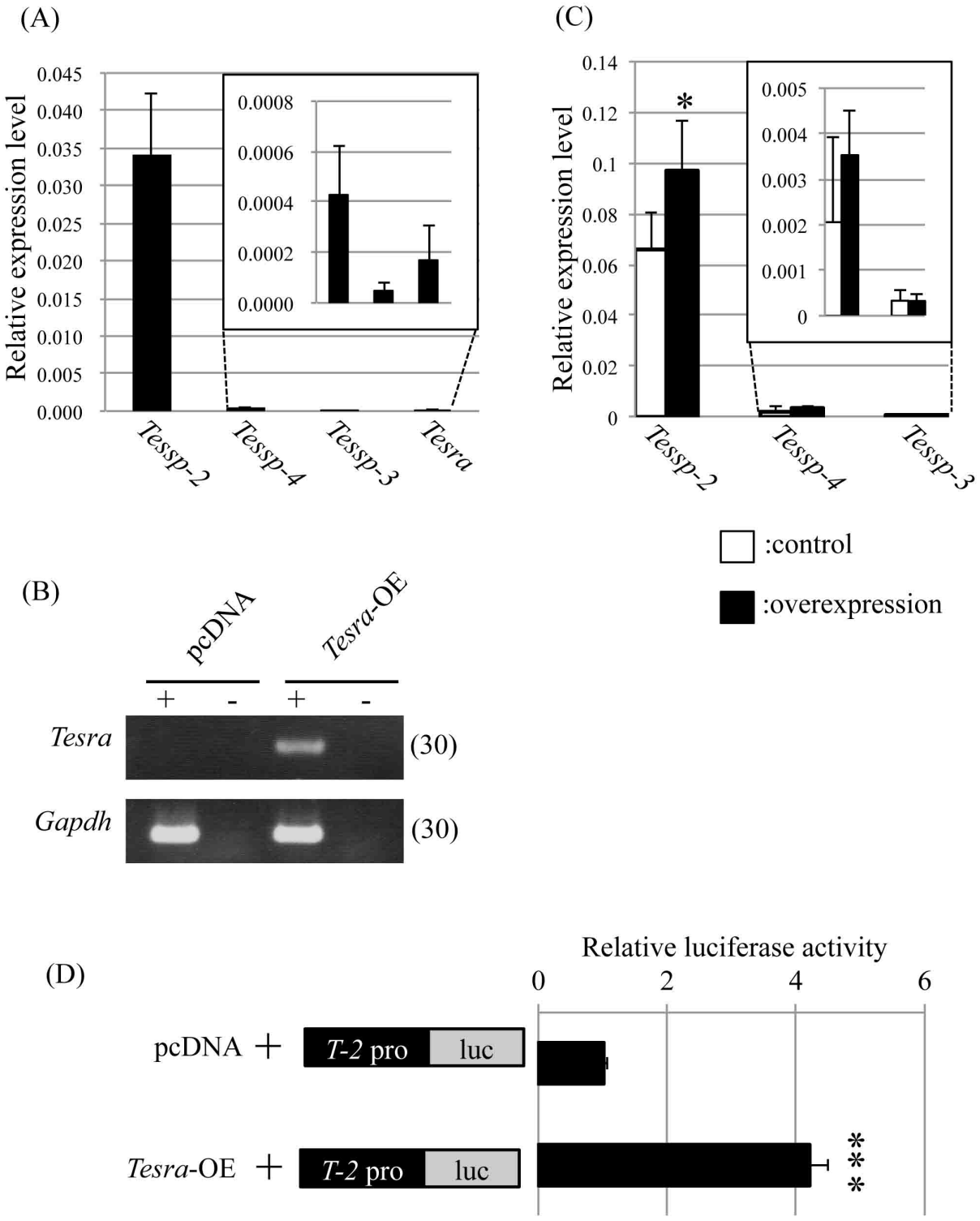

(A)
Relative expression level
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.0008
0.0006
0.0004
0.0002
0.0000
Tessp-2
Tessp-4
Tessp-3
Tesra
(C)
Relative expression level
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
0.005
0.004
0.003
0.002
0.001
0
Tessp-2
Tessp-4
Tessp-3
:control
:overexpression
(B)
pcDNA
Tesra-OE
+
-
+
-
Tesra
(30)
Gapdh
(30)
(D)
Relative luciferase activity
0
2
4
6
pcDNA +
T-2 pro
luc
Tesra-OE +
T-2 pro
luc
***

Figure 2-3. Overexpression of *Tesra* increases *Prss42/Tessp-2* expression. A: Endogenous expression of *Prss/Tessp* cluster genes and *Tesra* in Hepa1-6 cells. qRT-PCR was performed with total RNAs from Hepa1-6 cells. Reverse transcription was done by using the oligo(dT) primer, and *Aip* gene was examined as an internal control. Expression levels were normalized to *Aip*. Although the *Prss42/Tessp-2* level was much higher than the others, all transcripts examined were detected by this analysis, as shown in the inset. **B:** Successful overexpression of *Tesra*. Tesra-OE or pcDNA3.1 vector was transiently transfected into Hepa1-6 cells, and after the selection with G418, total RNA was purified from each sample. cDNA was synthesized with the oligo(dT) primer, and PCR was conducted to detect *Tesra* and *Gapdh* expression. The cycle number is presented in the parenthesis. A representative result out of three experiments is shown. We could not see any difference among the three data. The *Tesra* signal was observed only in the cells with Tesra-OE. **C:** Relative expression of *Prss/Tessp* mRNAs by overexpression of *Tesra*. qRT-PCR was performed for three *Prss/Tess*p cluster genes with the cDNAs prepared as above. All the data were normalized to *Aip*. The transient overexpression of *Tesra* significantly increased *Prss42/Tessp-2* expression in Hepa1-6 cells. The data are presented as mean ± S.D. from three independent experiments and were analyzed by Student's *t* test to compare the level of control with that of Tesra-OE. *P < 0.05 relative to the control. **D:** Luciferase assay in Hepa1-6 cells transfected with Tesra-OE. T-2pro-luc was transiently co-transfected with Tesra-OE or pcDNA3.1 into Hepa1-6 cells, and luciferase activity was measured 2 days later. Relative luciferase activity was presented as setting the activity with pcDNA3.1 to 1.0. *Tesra* overexpression significantly increased *Prss42/Tessp-2* promoter activity. The data are presented as mean ± S.D. from three independent experiments and were analyzed by Student's *t* test. ***P < 0.001 relative to the control.

Fig. 2-4

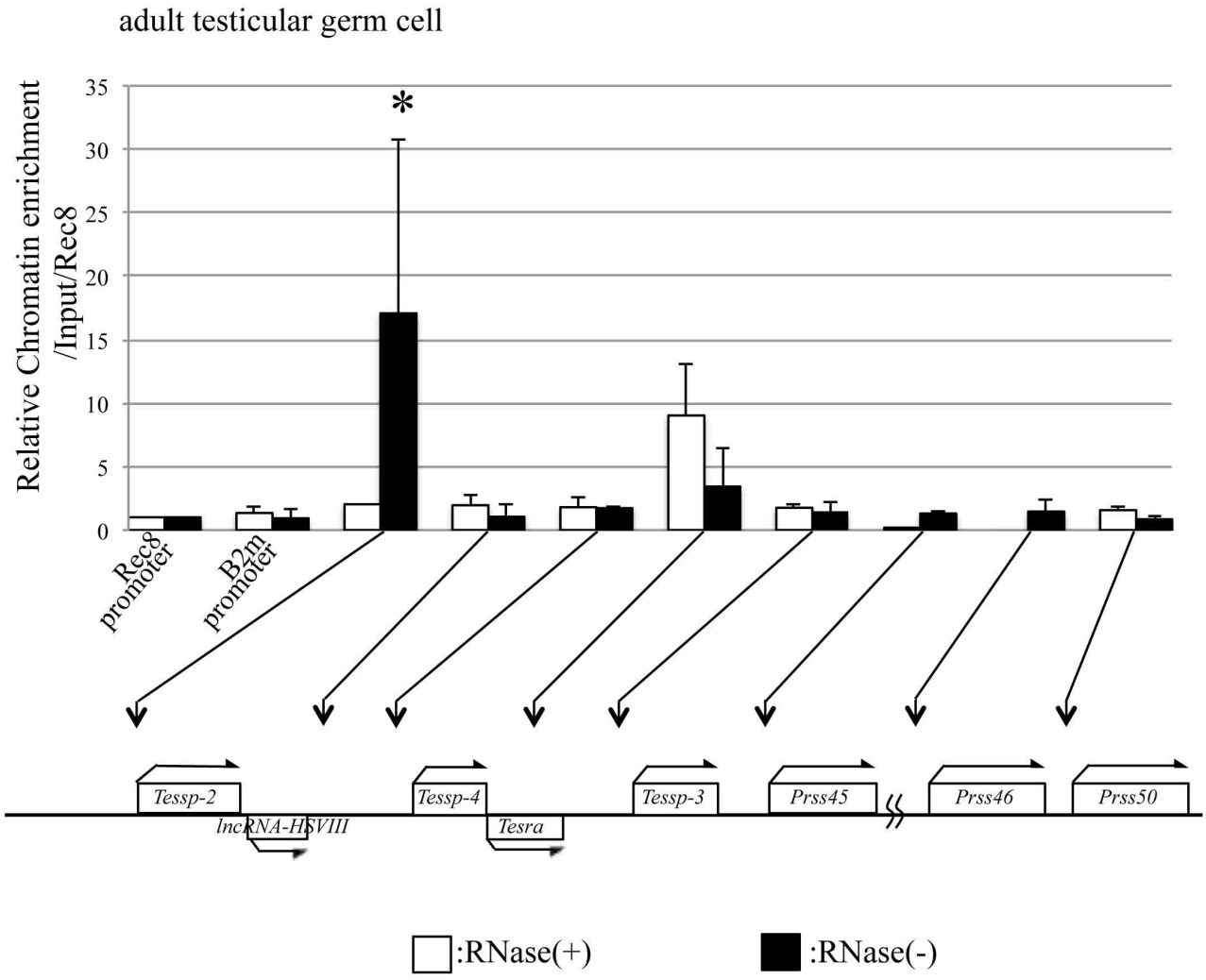
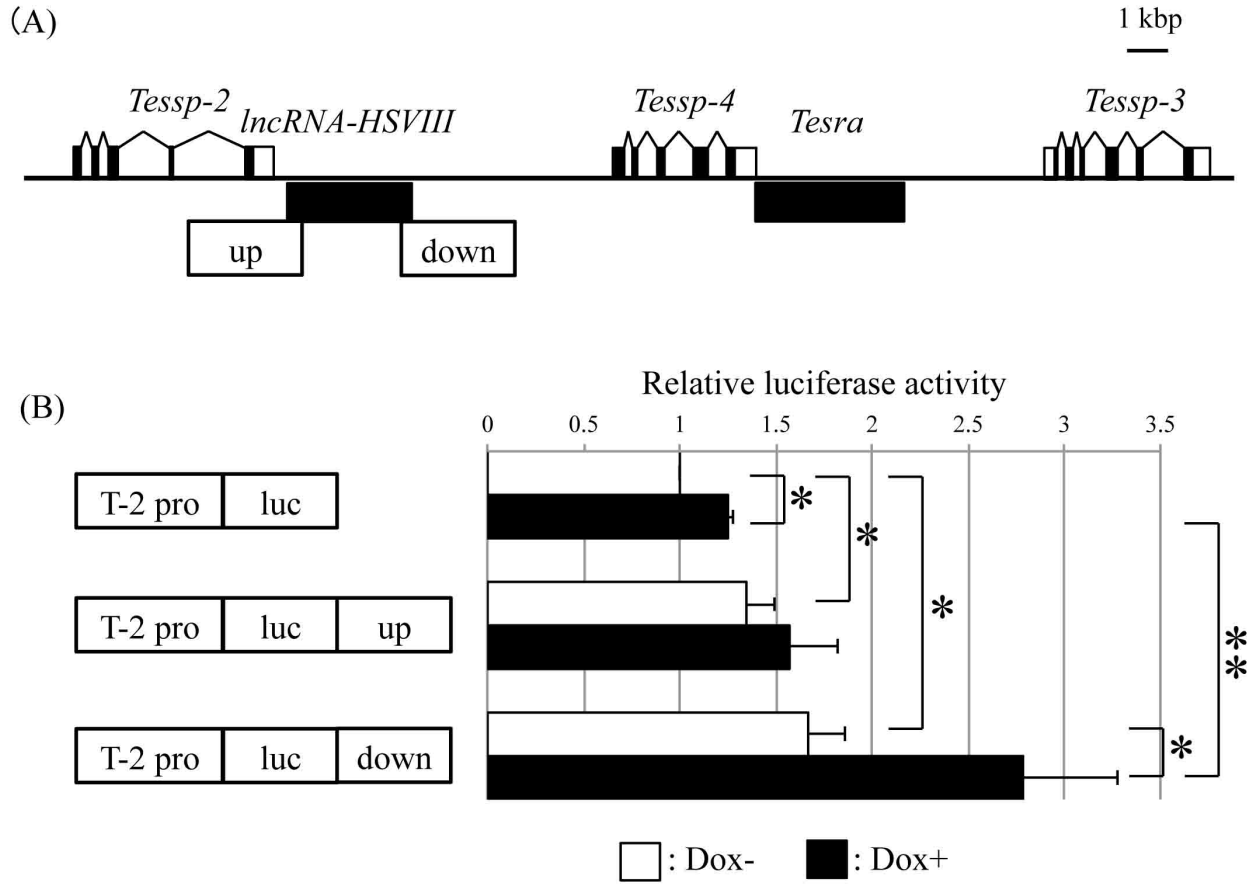

adult testicular germ cell
Relative Chromatin enrichment /Input/Rec8
35
30
25
20
15
10
5
0
*
Rec8 promoter
B2m promoter
Tessp-2
lncRNA-HSVIII
Tessp-4
Tesra
Tessp-3
Prss45
Prss46
Prss50
:RNase(+)
:RNase(-)

Figure 2-4. *Tesra* occupancy at the *Prss/Tessp* locus. ChIRP-qPCR for *Tesra* in mouse testicular germ cells were performed. Germ cells were collected from 21-22 days old mouse testes, and nuclear extracts were prepared and lysed. The sonicated chromatin was hybridized with biotinylated tiling oligo probes, and the bound chromatin was collected by streptavidin beads. The purified genome DNAs were investigated by qPCR. *Rec8* and *B2m* promoters that were located on different chromosomes from the *Prss/Tessp* cluster were amplified as negative controls. The value was normalized to that of Input sample, which was kept before hybridization, and further normalized to the level at the *Rec8* promoter (=1.0). The relative chromatin enrichment at each position is shown by black bars. White bars show the data from the experiment with RNase in hybridization buffer as a negative control. The position of amplicon is drawn below the graph. The data are presented as mean ± S.D. from three independent experiments and were analyzed by one-way ANOVA followed by Tukey's post hoc test. *P < 0.05 compared to other regions.

Fig. 2−5


(A)
1 kbp
Tessp-2
lncRNA-HSVIII
Tessp-4
Tesra
Tessp-3
up
down
(B)
Relative luciferase activity
0
0.5
1
1.5
2
2.5
3
3.5
T-2 pro
luc
T-2 pro
luc
up
T-2 pro
luc
down
*
*
*
*
**
: Dox-
: Dox+

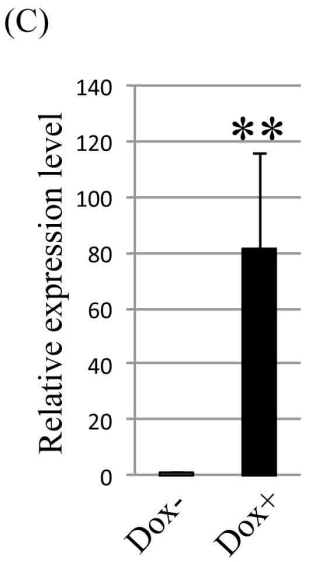

(C)
Relative expression level
140
120
100
80
60
40
20
0
**
Dox-
Dox+

Figure 2-5. *Tesra* co-operatively increases *Prss42/Tessp-2* promoter activity with the downstream enhancer. A: A schematic view of the *Prss/Tessp* locus indicating the positions of enhancers. The *Prss/Tessp* locus is depicted as in Fig. 2-1A. Two potential enhancers I detected in chapter 1 are indicated with white boxes marked as 'up' and 'down'. **B:** Luciferase assay in stable *Tesra*-inducible Hepa1-6 cells. I established stable Hepa1-6 cells that were responsible to Dox to induce *Tesra* transcription by the Tet-on system. Each construct indicated at left was transiently transfected into these cells. Dox was added 24 hours after the transfection, and luciferase activity was measured after another 24 hours. The activity in the cells with T-2pro-luc without Dox was set to 1.0. The data are presented as mean ± S.D. from three independent experiments, and the statistical significance was analyzed by one-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$. **$P < 0.01$. **C:** Successful induction of *Tesra* transcription in stable Hepa1-6 cells. Dox was added to the cells, and total RNAs were collected 24 hours later. qRT-PCR was performed as in Fig. 2-2D. *Tesra* transcription was significantly induced by the addition of Dox. ** $P < 0.01$ relative to Dox-.

Fig. 3

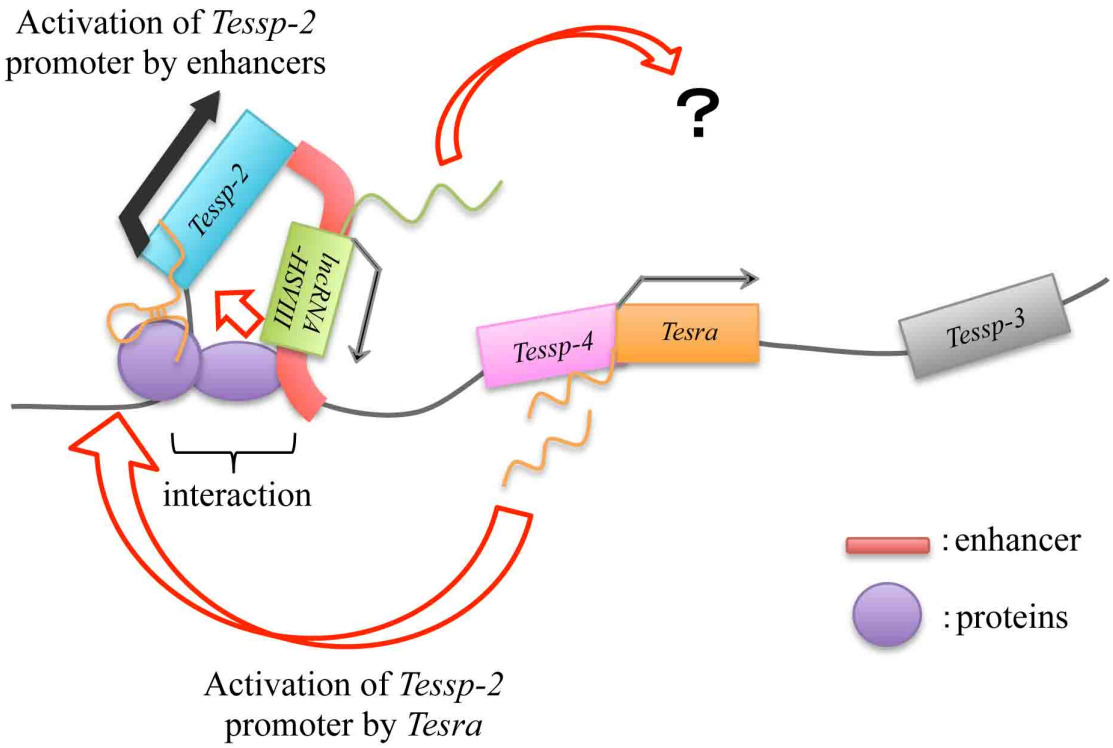


Figure 3. A model for *Prss42/Tessp-2* transcriptional activation. This figure indicates the hypothesized mechanism of *Prss42/Tessp-2* gene activation from my data. When spermatogonia divide into primary spermatocytes, the chromatin at the enhancer downstream of *lncRNA-HSVIII* begins to interact with that at the *Prss42/Tessp-2* promoter, and thereby the enhancer is allowed to activate *Prss42/Tessp-2* transcription. At the similar timing, *Tesra* is expressed and binds to the *Prss42/Tessp-2* promoter. The transcription level of *Prss42/Tessp-2* is substantially enhanced by these two elements.