A Testis-Specific Long Non-Coding RNA, IncRNA-Tcam1, Regulates Immune-Related Genes in Mouse Male Germ Cells

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Spermatogenesis is precisely controlled by hormones from the hypothalamus–pituitary–gonadal axis and testis-specific genes, but the regulatory mechanism is not fully understood. Recently, a large number of long non-coding RNAs (lncRNAs) are found to be transcribed at each stage of meiosis of male germ cells, and their functions in spermatogenesis have yet to be fully investigated. IncRNA-testicular cell adhesion molecule 1 (IncRNA-Tcam1) is a nuclear lncRNA which is specifically expressed in mouse male germ cells and presumed to play a role in gene regulation during meiosis. Here, we present the identification of potential target genes of IncRNA-Tcam1 using spermatocyte-derived GC-2spd(ts) cells. Initially, 55 target gene candidates were detected by RNA-sequencing of two GC-2spd(ts) cell clones that were stably transfected with transgenes to express IncRNA-Tcam1 at different levels. Expression of 21 genes of the candidates was found to be correlated with IncRNA-Tcam1 at 7–14 postnatal days, when IncRNA-Tcam1 expression was elevated. Subsequently, we examined expression levels of the 21 genes in other two GC-2spd(ts) clones, and 11 genes exhibited the correlation with IncRNA-Tcam1. Induction of IncRNA-Tcam1 transcription using the Tet-off system verified that six genes, Trim30a, Ifit3, Tgtp2, Ifi47, Oas1g, and Gbp3, were upregulated in GC-2spd(ts) cells, indicating that IncRNA-Tcam1 is responsible for the regulation of gene expression of the six genes. In addition, five of the six genes, namely, Ifit3, Tgtp2, Ifi47, Oas1g, and Gbp3, are immune response genes, and Trim30a is a negative regulator of immune response. Altogether, the present study suggests that IncRNA-Tcam1 is responsible for gene regulation for the immune response during spermatogenesis.

Keywords: long non-coding RNA, spermatogenesis, GC-2spd(ts) cells, gene activation, immune response

INTRODUCTION

Spermatogenesis is a process to generate spermatozoa, composed of meiosis and spermiogenesis (1, 2). In meiosis, some population of spermatogonia differentiate into spermatids, and the spermatids change their shapes to become spermatozoa through spermiogenesis. This process is controlled by the hypothalamus–pituitary–gonadal axis. The pituitary secretes gonadotropin in response to gonadotropin releasing hormone from hypothalamus, by which steroid production is stimulated in testis, leading to normal progression of spermatogenesis (3–5). In addition, many testis-specific
genes are activated in germ cells during meiosis and play important roles in spermatogenesis (6–14). However, the regulatory mechanism of spermatogenesis is not fully understood. To date, various long non-coding RNAs (lncRNAs) have been identified in the testis (15, 16), and some of them were shown to be upregulated by sex steroids (17–21). These findings suggest that lncRNAs are involved in the regulation of spermatogenesis. Nevertheless, the physiological significance of lncRNAs and the molecular mechanisms of their actions in the testis are largely unknown.

Long non-coding RNAs are molecules that are longer than 200 nucleotides and function without being translated (22). They are localized in nuclei, cytosols, and extracellular vesicles (23–25), and involved in various biological events such as development (26, 27), cell differentiation (28, 29), immune response (30, 31), and cell metabolism (32, 33). lncRNAs exert their effects via controlling gene expression at various levels, i.e., transcription, mRNA stability, and translation (22, 26, 34). In nuclei, lncRNAs are frequently involved in the transcriptional control of protein-coding genes (35). Thus, lncRNAs are presumed to be essential for transcriptional regulation of meiotic genes during spermatogenesis, while biological functions of testicular lncRNAs have not been well investigated.

We have studied the mechanism of gene activation during meiosis by using the mouse testicular cell adhesion molecule 1 (Tcam1) locus as a model. The Tcam1 gene is specifically expressed in spermatocytes and encodes a cell adhesion molecule, which is dispensable for sperm generation (36, 37). We previously identified a dual promoter–enhancer, which enhanced the Tcam1 gene transcription and drove the SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 2 (Smarcd2) gene and lncRNA-Tcam1 (38). lncRNA-Tcam1 was a novel testis-specific lncRNA which was 2.4-kb nucleotide long and localized to nuclei of germ cells but not to Leydig and Sertoli cells (38). Such germ cell-specific localization suggests that lncRNA-Tcam1 plays a role in specific gene activation during meiosis.

To study molecular mechanisms in various types of cells, the in vitro cell culture system is useful, and some cell lines were established from male germ cells (39). The GC-2spd(ts) cell line was established from mouse spermatocytes using the SV40 large T antigen and the temperature-sensitive P53 mutant gene (40), and was widely used due to their ability of differentiation. After T antigen and the temperature-sensitive P53 mutant gene (40), was established from mouse spermatocytes using the SV40 large in vivo meiosis.

Figure 1A (promoter). 

### MATERIALS AND METHODS

#### Animals

The mice (C57/BL6) were maintained at 25°C with a photoperiod of 14:10 h light–dark with free access to food and water. Testes were isolated from 7- to 56-day-old mice and stored at −80°C until use. Experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

#### Cell Culture

GC-2spd(ts) and its derivatives were cultured in Dulbecco Modified Eagle medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml l-glutamine (Invitrogen, Carlsbad, CA, USA) (38, 40). We used two types of GC-2spd(ts)-derived cell clones: lncRNA-6.9kb-EGFP clones that contained the intact sequence of the lncRNA-Tcam1 locus (full sequence) and ΔCNS1-lncRNA-EGFP clones that lacked the lncRNA-Tcam1 promoter (Δpromoter) (Figure 1A).

#### RNA Preparation and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Isolation of total RNAs, treatment with DNase, and qRT-PCR were done as previously described (38, 49). To detect lncRNA-Tcam1, we used KOD SYBR qPCR Mix (Toyobo, Osaka, Japan), and the other transcripts were measured with Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primer pairs are listed in Table 1.

#### RNA-sequencing (RNA-seq) Analysis

Total RNAs were isolated from a lncRNA-6.9kb-EGFP clone (full sequence) and ΔCNS1-lncRNA-EGFP clone (Δpromoter) using ISOGEN II (Nippongene, Tokyo, Japan) according to the manufacturer's instruction. The RNAs were further purified by phenol/chloroform–isoamylalcohol extraction and ethanol precipitation, and treated with TurboDNase (Applied Biosystems). The quality of the RNA samples was evaluated using BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA) with RNA6000 Nano Chip. A 500-ng aliquot of total RNA from each sample was used to construct cDNA libraries using TruSeq Stranded mRNA Sample preparation kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. The resulting cDNA library was validated using BioAnalyzer with DNA1000 Chip and quantified using Cycleave PCR Quantification Kit (Takara Bio Inc., Otsu, Japan). 101-cycle of single-end sequencing was performed using HiSeq1500 (Illumina) in the rapid mode. Total reads were extracted with CASAVA v1.8.2 (Illumina). Then, PCR duplicates, adaptor sequences and low quality reads were removed from the extracted reads as follows. Briefly, if the first 10 bases of the two reads were identical and the entire reads exhibited >90% similarity, the reads were considered PCR duplicates. Remaining reads were then aligned with Bowtie version 2.2.3 to the mouse genome, mm10, which was downloaded from UCSC (https://genome.ucsc.edu/). The expression level of each gene was calculated as gene-specific reads per million total reads (RPM).

#### Plasmid Construction

A PiggyBac-based plasmid for induction of lncRNA-Tcam1 transcription was generated as follows. A full length of lncRNA-Tcam1
Figure 1 A strategy to identify potential target genes of long non-coding RNA-testicular cell adhesion molecule 1 (lncRNA-Tcam1). (A) Constructs used for establishing GC-2spd(ts) cell clones that expressed lncRNA-Tcam1 at different levels. A 6.9-kb sequence encompassing the upstream and downstream regions of lncRNA-Tcam1 was obtained from a mouse BAC clone, and connected to the enhanced green fluorescent protein (EGFP) gene (full sequence). A 0.8-kb sequence corresponding to the lncRNA-Tcam1 promoter was deleted by restriction digestion (Δprom). The reporter EGFP gene was used to investigate the transcriptional regulation activity of the lncRNA promoter sequence in our previous study. Each construct was transfected into GC-2spd(ts) cells, and stable cell clones were established by the limited dilution method.

(B) Expression of lncRNA-Tcam1 in GC-2spd-Full and GC-2spd-Δprom. GC-2spd-Δprom was derived from the cells transfected with "Δprom" and used as a clone expressing lncRNA-Tcam1 at a low level. GC-2spd-Full was a stably transfected clone with "full sequence" and used as the cells expressing lncRNA-Tcam1 at a high level. Total RNA was isolated from each clone, and the expression level of lncRNA-Tcam1 was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The value was normalized to the Gapdh mRNA level, and the level in GC-2spd-Δprom was set to 1.0. The data are presented as mean value ± SD from three independent experiments.

(C) An outline of the screening process to identify potential target genes of lncRNA-Tcam1 in this study.

was amplified by PCR with mouse genome DNA and KOD FX Neo (Toyobo) using a primer pair, 5′-CCGGAGGAGCGGAA-3′ and 5′-CACCCGAAACAGCTTTAAT-3′. We used the genome DNA as a template because lncRNA-Tcam1 contained no intron. The product was subcloned into a pBluescript II vector (Stratagene, La Jolla, CA, USA) at EcoRV site, and the resulting plasmid was checked by DNA sequencing method. This plasmid was digested with SalI, blunted by T4 DNA polymerase (Takara), and further digested with NotI. The lncRNA-Tcam1 fragment was purified and inserted into pPBhCMV*1-cHA-pPA at the NotI/blunted XhoI site. The completed plasmid was checked by DNA sequencing and named pPBhCMV*1-lncTcam1-pPA. All plasmids for establishing the Tet-off system were kindly gifted by Dr. Kazuhiro Murakami (50).
Establishment of the Tet-Off System and Induction of IncRNA-Tcam1 Transcription

GC-2spd(ts) cells were co-transfected with pBBlCMV*1-IncTcam1-pPA, pPBCAGT-AIN, pPyCAG-Base, and pKO-Select-Puro plasmids using Genejuice transfection reagent (Merck, Darmstadt, Germany) and selected with 3 µg/ml puromycin for 14 days. The selected cells were kept in the presence of 1 µg/ml doxycycline (Dox). For inducing IncRNA-Tcam1 transcription, the cells were spread onto 35-mm dishes, and Dox was removed on the next day.

Statistical Analysis

The results were expressed as means ± SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s test (Figures 2A, 3B, C) or by Student’s t-test (Figures 2B, C, 3A, and 4B) using Microsoft Excel statistical analysis functions (Microsoft Corporation, Redmond, WA, USA).

RESULTS

RNA-seq with GC-2spd(ts) Cell Clones Expressing IncRNA-Tcam1 at Different Levels

To identify potential target genes that were regulated by IncRNA-Tcam1, we used GC-2spd(ts) cell clones we established in a previous study (38). In these lines, we stably transfected a construct, which contained a 6.9-kb sequence encompassing the Tcam1 promoter and the IncRNA-Tcam1 region (full sequence)

or the same sequence without the IncRNA-Tcam1 promoter (Δpromoter), connected to the enhanced green fluorescent protein (EGFP) gene (Figure 1A). IncRNA-Tcam1 was expressed at higher levels in the clones with “full sequence” than those with “Δpromoter” (38), and hereafter, we designated “GC-2spd-Full” and “GC-2spd-Δprom,” respectively. We chose a representative cell clone from each cell line, and the GC-2spd-Full and GC-2spd-Δprom clones showed 63-fold difference in expression levels of IncRNA-Tcam1 by qRT-PCR (Figure 1B). We expected that target genes of this lncRNA should be expressed at different levels between them.

To determine the genes that were differentially expressed between GC-2spd-Full and GC-2spd-Δprom, we performed the RNA-seq analysis. 101-cycle of single-end sequencing using HiSeq 1500 yielded 13,054,029 and 13,194,037 reads for GC-2spd-Full and GC-2spd-Δprom, respectively, and 92.25% of which was mapped to the mouse genome (mm10). The resultant fastq files were deposited on NCBI SRA database (accession no. SRR5435658 and SRR5435659).

Subsequently, we compared the gene expression levels between GC-2spd-Full and GC-2spd-Δprom, and picked protein-coding genes that were expressed at lower levels than 0.1-fold in GC-2spd-Full as candidate genes negatively regulated by IncRNA-Tcam1, and those expressed at higher levels than fivefold in GC-2spd-Full as positively regulated genes. We then selected the gene whose RPMK value was higher than 1.5 in GC-2spd-Δprom or GC-2spd-Full. As a result, 29 genes were chosen as candidates potentially suppressed by IncRNA-Tcam1, and 27 genes as those activated by the lncRNA. However, after this selection, we found that one potentially activated gene was

TABLE 1 | Oligonucleotide primers used in this study.

| Designation | Forward | Reverse |
|-------------|---------|---------|
| IncRNA-Tcam1 | 5′-GACTGTCTGGCAAGAATGA-3′ | 5′-GAACCAAGAACAGTCTGAA-3′ |
| Aip | 5′-GGAGCAGCTTCCAAGAAGC-3′ | 5′-CTTGCAGGCTGCGAAAGT-3′ |
| Gapdh | 5′-TGACCAACCACTGCTTGAAG-3′ | 5′-GGCCATGAGCTTGCAGTCAGAG-3′ |
| H19 | 5′-TGGGAAATGAAATGAAAGC-3′ | 5′-GTGATGAGCAGAGAGAAGAAG-3′ |
| Ren1 | 5′-ATCCTTCATTCTCAGTCTA-3′ | 5′-ACCCCTGGCTCAACACAGA-3′ |
| Igfbp5 | 5′-CTGACCCCTCTAACCCTCCTT-3′ | 5′-TGGAGCAGAATTTCTGCTTGA-3′ |
| Gpx7 | 5′-GTTGACCCAGAAGGAAAC-3′ | 5′-GGGAATGACCTTCAAGCTGAGAG-3′ |
| Bmhc | 5′-GACCAAGCAGGCTGAGGTTT-3′ | 5′-CCCAAGCATCTTCCACCC-3′ |
| A431a | 5′-AAAGACTGGGACAGGAAAGT-3′ | 5′-GTCAGACCTCCACCC-3′ |
| Igf1 | 5′-AAAGAGCAGACGCAGGCTAT-3′ | 5′-GCTGAGGAGGAAATCATTAT-3′ |
| Oas2 | 5′-CTACCTGCCACGGAGTCAAA-3′ | 5′-TGGACCTTTCCAAGGGTGTA-3′ |
| Trim30a | 5′-GGACAGGTATCTTCTCCTT-3′ | 5′-GTCCTTCTGTTGTTATCTG-3′ |
| Ifit3 | 5′-CCAGACAGCCAGAAAGCAGAT-3′ | 5′-GCATCTTCCACCC-3′ |
| Hox13 | 5′-GGAAACACCGAGGCTGACTG-3′ | 5′-CTTCACCCACCC-3′ |
| Tgfp2 | 5′-ATGCTCTGTAATGTTGAG-3′ | 5′-GCAACTGACCAAGCAGAT-3′ |
| Mx2 | 5′-TCTAAAGAACACCTCCATT-3′ | 5′-GCTGAGGTGCTGCTCTT-3′ |
| Fabp5 | 5′-ACGGAGAGAGAGACAGCAG-3′ | 5′-GCAAGTGGAGTTGCTTCA-3′ |
| Ifit47 | 5′-GTTGAGAAAGACCCGGCTAT-3′ | 5′-ATGCTCTGTCGCTTCTGAT-3′ |
| Enpp5 | 5′-CCTCTTCTGCCCTTCTTCTT-3′ | 5′-AGCGAGTGAGGATAGTAG-3′ |
| Oas1g | 5′-CCAGAGAGAGGAGGTTGAG-3′ | 5′-TCAGAGGAGTTGGTTGAT-3′ |
| Usp18 | 5′-CTGGAGTCCAGGACAGCAAA-3′ | 5′-ACCAAGAAGCCGATCCCAA-3′ |
| Cgbp3 | 5′-GGATCTTTGAGGAGATAC-3′ | 5′-ATACCCCTGTTGTTGATT-3′ |
| Ifit44 | 5′-GCCCTTGAGTGAGTTCGCT-3′ | 5′-GCCAGTTTATCTGCTTGAA-3′ |
| Ifit1 | 5′-GATTCTGCTGCTGCTGCTGAAA-3′ | 5′-AGGAACCTGGACCTTCTG-3′ |
Figure 2 | Expression of long non-coding RNA-testicular cell adhesion molecule 1 (lncRNA-Tcam1) and its target gene candidates in postnatal testes. (A) lncRNA-Tcam1 expression in mouse testes at various postnatal stages. Total RNAs were purified from whole testes of mice at indicated ages. Expression levels of lncRNA-Tcam1 were measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as in Figure 1B, and the data were normalized to the housekeeping Aip gene. The value at day 7 was set to 1.0. (B) Expression of five candidate genes that were presumed to be suppressed by lncRNA-Tcam1 in testes of 7- and 14-day-old mice. qRT-PCR was performed, and the data were normalized to Aip. The level at day 7 was set to 1.0. Light gray bars represent expression levels at 7 days of age, and light black bars indicate those at 14 days. (C) Expression of 16 candidate genes that were presumed to be activated by lncRNA-Tcam1. qRT-PCR was performed and the data were normalized as in (B). All experiments were performed four times with two different RNA sets from testes, and the averages ± SD are indicated. The statistical significance was analyzed by one-way analysis of variance followed by Dunnett’s test in (A) and by Student’s t-test in (B,C). *P < 0.05, **P < 0.01 compared to day 7.

not detected in GC-2spd-Full by qRT-PCR, and eliminated it from candidate genes. Consequently, a total of 55 genes (29 potentially suppressed and 26 activated genes, Tables S1 and S2 in Supplementary Material) were subject to further analysis. An outline of the screening of candidate genes is shown in Figure 1C.
Expression of Candidate Genes in Postnatal Testes

The target genes should be expressed correlatively to IncRNA-Tcam1 in the testis. We thus examined the expression of these potential target genes during postnatal testis development. We collected testes at 7–56 days after birth and measured the IncRNA-Tcam1 level by qRT-PCR. The transcript of IncRNA-Tcam1 was detected in all samples, and a significant increase was observed...
between day 7 and day 14 after birth (Figure 2A). The absence of lncRNA-Tcam1 expression in somatic cells in the mouse testis (38) led to the presumption that the transcription of lncRNA-Tcam1 is initiated in spermatogonia and increased in primary spermatocytes. Then, we examined expression levels of the 55 candidate genes (29 potentially downregulated and 26 upregulated) in the testis at 7 days and 14 days after birth by qRT-PCR, to compare their levels in spermatogonia and primary spermatocytes. Among the genes that might be suppressed by lncRNA-Tcam1, 5 genes showed significant decreases during this period (Figure 2B), and 16 genes out of potentially activated candidates exhibited significant increases (Figure 2C). The expression patterns of genes that were not correlated with lncRNA-Tcam1 is presented in Figures S1 and S2 in Supplementary Material. Signals for four downregulated candidates, Cyclin D2 (Ccn2), CD55 molecule, decay accelerating factor for complement (Cd55), Syndecan 3 (Sdc3), and uc009oek.1, and an upregulated candidate, 2′-5′ oligoadenylate synthetase 3 (Oas3), were not detected by this analysis. From these data, 21 genes (5 genes down and 16 genes up) correlated with in vivo lncRNA-Tcam1 expression were forwarded to the next analysis as target gene candidates.

Expression of Candidate Genes in GC-2spd(ts) Cell Clones Different from Those Used for RNA-seq

To further validate the RNA-seq and postnatal testis data, the expression was also examined using GC-2spd(ts) clones that were different from those used for RNA-seq. This would help us to select the real targets, because the properties might be different between cell clones as a result of the process to establish stable cells and pick the clones. qRT-PCR demonstrated that lncRNA-Tcam1 was expressed at a 3.5-fold higher level in GC-2spd-Full than in GC-2spd-Δprom (Figure 3A). We assessed the expression of 21 candidate genes using these two clones and GC-2spd(ts) cells without being transfected. By comparing to cells without being transfected, false-positive genes (due to the establishment of cell clones) could be excluded. Among five genes potentially suppressed by lncRNA-Tcam1, H19, imprinted maternally expressed transcript (H19), Insulin-like growth factor binding protein 5 (Igfbp5), and Brain expressed myelocytomatosis oncogene (Bmyc) were expressed at much higher levels in GC-2spd-Δprom than in GC-2spd-Full, but they were expressed at very low levels in GC-2spd(ts) cells without transfection (Figure 3B), indicating that these three genes were artifacts. Expression of Renin 1 structural (Ren1) and Glutathione peroxidase 7 (Gpx7) genes was positively correlated with lncRNA-Tcam1 (Figure 3B), which was inconsistent with the genes suppressed by lncRNA-Tcam1. Therefore, the five genes were unlikely to be targets of lncRNA-Tcam1.

Among 16 genes, expression levels of five genes [2′-5′ oligoadenylate synthetase 2 (Oas2), Homeobox D13 (Hoxd13), MX dynamin-like GTase 2 (Mx2), Fatty acid binding protein 5, epidermal (Fabp5), and Interferon-induced protein wit tetratricopeptide repeats 1 (Ifi11)] were not significantly higher in GC-2spd-Full than in GC-2spd(ts) cells (Figure 3C), excluding the possibility that these genes were targets of lncRNA-Tcam1. On the other hand, 11 genes were expressed at significantly higher levels in GC-2spd-Full than in GC-2spd(ts) cells, and their expression was higher in GC-2spd-Full than in GC-2spd-Δprom (Figure 3C). Collectively, these expression profiles detected 11 potential genes upregulated by lncRNA-Tcam1 and they were subjected to further analysis.

Identification of Genes Upregulated by lncRNA-Tcam1

We finally examined whether lncRNA-Tcam1 could activate the 11 candidate genes in the Tet-off system. We constructed the plasmid in which the hCMV*1 promoter induced lncRNA-Tcam1 transcription in response to the Dox removal, and stably transsected GC-2spd(ts) cells with it. qRT-PCR of the stable transfectants confirmed that the expression of lncRNA-Tcam1 was 57-fold induced exclusively at 48 h after removal of Dox (Figure 4A).

We examined expression levels of the 11 candidate genes 48 h after the Dox removal by qRT-PCR. We set the value from the control cells that were cultured for 48 h with Dox to 1.0 and calculated the levels of candidates. six genes, Tripartite motif-containing 30A (Trim30a), Interferon-induced protein wit tetratricopeptide repeats 3 (Ifi3), T cell-specific GTase 2 (Tgtp2), Interferon gamma inducible protein 47 (Ifi47), 2′-5′ oligoadenylate synthetase 1G (Oas1g), and Guanylate binding protein 3 (Gbp3), exhibited significantly higher expression in the cells without Dox, and their fold-increases were 1.46, 3.37, 2.43, 1.44, 1.49, and 1.69, respectively (Figure 4B). We confirmed significant levels of expression of the six genes in the germ cell fraction from adult mouse testes (Table S3 in Supplementary Material). Interestingly, five genes of the final six candidates, namely, Ifi3, Tgtp2, Ifi47, Oas1g, and Gbp3, were immune response genes, and Trim30a was a negative-feedback regulator of immune response (Table 2). Collectively, these data indicated that lncRNA-Tcam1 is responsible for the regulation of some immune response pathways during spermatogenesis.

DISCUSSION

Among various mouse and human tissues, the testis contains large numbers of lncRNAs (15, 16), as evidenced by recent transcriptomic analyses identifying many lncRNAs in different postnatal testes or fractionated germ cells (51–53). However, their functions in spermatogenesis are largely unknown in mammals, and we revealed the upregulation of gene expression of immune-related genes by mouse male germ cell-specific lncRNA-Tcam1 using spermatocyte-derived GC-2spd(ts) cells as a model. We performed RNA-seq of GC-2spd(ts) cell clones that expressed lncRNA-Tcam1 at different levels and selected 55 genes as initial candidates of its targets. Then, we investigated (1) their expression in testes at different developmental stages, (2) their expression in GC-2spd(ts) clones different from those used for RNA-seq, and (3) the response to induction of lncRNA-Tcam1 transcription by the Tet-off system. Eventually, these analyses detected six immune-related genes, Trim30a, Ifi3, Tgtp2, Ifi47, Oas1g, and Gbp3 that were upregulated by lncRNA-Tcam1. Taken
Figure 4 | Expression of target gene candidates in response to induction of long non-coding RNA-Tcam1 (lncRNA-Tcam1) transcription by the Tet-off system.

(A) Induction of lncRNA-Tcam1 transcription by the Tet-off system. GC-2spd(ts) cells were transfected with the construct for lncRNA-Tcam1 overexpression in response to the doxycycline (Dox) removal. After the selection of successfully transfected cells with puromycin, they were cultured in the presence (Dox+) or absence of Dox (Dox−) for 24 or 48 h. The cells were collected, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to measure the lncRNA-Tcam1 level using Gapdh as an internal control. The data represent the means ± SD from three independent experiments. A dramatic increase of lncRNA-Tcam1 transcription was observed in the Dox− sample at 48 h after the induction.

(B) Expression of candidate genes at 48 h after the induction. qRT-PCR was performed, and the data were normalized to Gapdh. The values from Dox− samples were further normalized to those from Dox+ samples and expressed as fold-increases. The data represents the means ± SD from five independent experiments. The statistical significance was analyzed by Student’s t-test. *P < 0.05, **P < 0.01 compared to Dox+ samples.
The physiological significance of some of the target genes in immune defense has been demonstrated by several studies. Interleukin 17a was elevated by infection of rhinovirus in the lung, and led to upregulation of the Oas1g gene, which caused the degradation of viral RNAs (64). Dengue virus infection in lung epithelial cells upregulated the Ifi47 gene, which suppressed the virus production (65). In dendritic cells, infection of Listeria monocytogenes increased expression of Tgtp2 and Oas1g genes to eventually reduce the bacterial titer (66). All of these indicate that the genes are responsive to various types of infection and contribute to immune defense in vivo, which suggests that they also play important roles in the immune response in the testis.

The testis is an immunoprivileged tissue and has evolved the innate immune system (67, 68). The innate immunity involves the interferon response, and key molecules for that are Toll-like receptors (TLRs). Activation of TLRs induces the interferon production and increases expression of interferon-responsive genes (67). Because mouse spermatogonia and primary spermatocytes constitutively express one of the TLR genes, Tlr3, and interferon receptor genes (69, 70), TLR3-mediated immune pathway is likely to be active in the testis. Notably, the five immune responsive genes are known to be responsive to interferon, so they may be activated through the TLR3 pathway in germ cells when the testis is infected by some pathogens. Therefore, IncRNA-Tcam1 may regulate some innate immune responses by controlling the interferon-responsive genes during meiosis.

It is now well known that a wide variety of IncRNAs are expressed in the testis (15, 16, 71), but their functional investigation has just begun. A few studies have thus far indicated biological roles of IncRNAs in self-renewal of spermatogonia (72), regulation of a signaling pathway (73), and progression of meiosis (74) during spermatogenesis. We originally revealed the involvement of a testis-specific IncRNA, IncRNA-Tcam1, in the regulation of immune-related genes in mouse spermatocyte-derived cells, and thus, the present study paves the way for investigating novel immune molecular mechanisms underpinning spermatogenesis via the endocrine system.

**ETHICS STATEMENT**

Experimental procedures used in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

**AUTHOR CONTRIBUTIONS**

MK and AK designed the study. MK, KO, SM, and AS performed the experiments. MK, KO, SM, AS, HS, and AK analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/article/10.3389/fendo.2017.00299/full#supplementary-material.

FIGURE S1 | Expression of target gene candidates downregulated by lncRNA-Tcam1 in postnatal testes. Total RNAs were isolated from 7- and 14-day-old mouse testes, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed for the candidate genes that might be downregulated by lncRNA-Tcam1. All the data were normalized to the value of Aip gene. Gray and light black bars represent the data in 7- and 14-day-old testes, respectively. None of the indicated genes showed inverse correlation to lncRNA-Tcam1.

FIGURE S2 | Expression of target gene candidates upregulated by lncRNA-Tcam1 in postnatal testes. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed, and the data are shown as in Supplemental Figure S1. None of the indicated genes exhibited positive correlation to lncRNA-Tcam1.

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