Impairment of Pachytene Spermatogenesis in Dmrt7 Deficient Mice, Possibly Causing Meiotic Arrest

Shiori Date,* Osamu Nozawa,* Hiroaki Inoue, Shizuhiko HideMA, and Katsuhiko Nishimiroy

Laboratory of Molecular Biology Graduate School of Agricultural Science, Tohoku University,
1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan

Received January 17, 2012; Accepted May 31, 2012; Online Publication, September 7, 2012
[doi:10.1271/bbb.120024]

Although Dmrt7 has been reported to be essential for male spermatogenesis, the molecular mechanism underlying pachytene spermatogenesis by Dmrt7 is not known. In the present study, by detailed analysis of Dmrt7 protein distribution in spermatocytes in the first wave of spermatogenesis, we clarified the profile of Dmrt7 expression and localization in pachytene spermatogenesis. Dmrt7-deficient spermatocytes were arrested in the pachytene stage, followed by apoptosis. We analyzed to determine whether every event in the spermatogenesis at the Dmrt7-deficient mice progressed normally, because in several gene knockout mice with spermatogenic arrest described in the previous reports impairments of these events often appeared. Mutant mice showed normal synopsis and XY body formation, while impairment of meiotic sex chromosome inactivation (MSCI), decreased expression of backup genes, and increased expression of retrotransposons indicated incomplete meiotic recombination.

Key words: spermatogenesis; male infertility; pachytene; spermatogenic arrest; Dmrt7

Among phyla, a number of mechanisms of sex determination and differentiation have been identified, and there are a few genes with conserved functions for regulation of sex differentiation.

The function of Dmrt7 in Drosophila melanogaster and male abnormal-3 (Mab-3) in Caenorhabditis elegans are well-known genes with conserved DNA binding finger domains.1) In flies, the sex-specific isoforms of Dsx (DsxM and DsxF) regulate most somatic sexual dimorphism. Male C. elegans mutants in Mab-3 showed defects in male genital development,2,3) a phenotype similar to male flies lacking DsxM function. DsxM (but not DsxF), the male isoform of the Dsx gene, partially compensated for the loss of mab-3 in C. elegans,4) indicating that the function was compatible and that the sex determination mechanism in invertebrates might be conserved. DM domain-containing genes are also present in vertebrates, and are called Dmrt (doublesex and mab-3 related transcription factors). As for those in D. melanogaster and C. elegans, some of the Dmrt genes that function in sex determination have been revealed in vertebrates. For example, in Oryzias latipes, mutations in the Dmy gene,5) in which the DM domain is conserved, cause male sex reversal, and in humans, several cases of deletion at or near the cluster of the DM domain-containing genes (Dmrt1, Dmrt2, and Dmrt3) on chromosome 9, caused XY sex reversal.5) In mice, to date 8 family members, from Dmrt1 to Dmrt8, have been identified.6) Mouse Dmrt7, the seventh member of the Dmrt family, was first reported in 2003,7) and expression analysis of the gene in various organs and tissues suggested that expression of it was limited to germ cells of the testis and somatic cells of ovary, suggesting that Dmrt7 can be categorized as a gene with highly specific expression in the gonads of both sexes.7)

Dmrt7 deficient mice have been generated by two groups.8,9) They found that Dmrt7 mutant males were infertile, with spermatogenic arrest at the pachytene stage, while the mutant females showed normal fertility.8,9) Another group reported that Dmrt7 localized in the XY body, and they suspected that Dmrt7 was related to histone modification of the sex chromosome,9) but their analysis was carried out using diploctene spermatocytes after pachytene arrest in spermatogenesis. They explained that pachytene arrest and abnormal sex chromatin might be two independent consequences of Dmrt7 loss. Recently, NF-Y was reported as possible transcriptional factor that binds to the CCAAT box promoter of Dmrt7.10) Thus, the function of Dmrt7 remains a mystery.

Materials and Methods

Animals. C57BL/6 and Dmrt7 deficient mice of mixed genetic background (C57BL/6 and 129/Sv)3) were housed on a 12:12 light-dark cycle. All experiments complied with the Guidelines for the Care and Use of Laboratory Animals of Tohoku University. The animals were euthanized by cervical dislocation and then the tissues were isolated.

Western blotting. We obtained nuclear extract proteins from the testes as previously described.11) The Western blotting procedure was as follows: the protein concentrations were determined by the Bradford method, and equal amounts (100 μg) of protein were loaded and separated by electrophoresis on 12.5% sodium-dodecyl-sulfate polyacrylamide gels. The separated proteins were transferred to polyvinylidene difluoride membranes and blocked for 1 h at 37°C with 5% fat-free milk in TRIS-buffered saline solution (TBS, 0.05 M, pH 7.4). Then, 1/500 diluted Dmrt7 antibody for nuclear extract proteins from mouse testes, diluted in 1% fat-free milk solution, were added,
followed by 2h of incubation at room temperature. Diluted horse-radish-peroxidase-conjugated goat anti-rabbit IgG antibody (1/1500) was added, and the membranes were incubated for a further 2h at room temperature. The targeted protein bands were visualized using an enhanced ECL Plus Western Blotting Detection System (GE Healthcare, USA).

### Histological analysis of testes.
We obtained tissue sections as previously described. The mouse testes were fixed in Bouin’s solution overnight, embedded in paraffin for 2h, sectioned (5μm) and stained with hematoxylin and eosin or periodic acid-Schiff (PAS).

### Immunostaining.
We obtained tissue sections as previously described. For immunohistochemical analysis, antigen activation was achieved by incubating de-waxed slides in 1 μm EDTA (pH 9.0) for 15 min at 90°C, and then blocking for 1h with 5% normal goat serum or 1% BSA in TBS. Primary antibodies were diluted 1/200-1/500 with 0.1% BSA in TBS and stored overnight at 4°C prior to detection with secondary antibodies. The nuclei were counterstained with DAPI. For immunofluorescence staining, the nuclei were counterstained with DAPI.

### Metiotic chromosome-spread preparation and fluorescent immunostaining.
We obtained nuclear spreads by a method previously described. We obtained tissue sections as previously described. For immunohistochemical analysis, antigen activation was achieved by incubating de-waxed slides in 1 μm EDTA (pH 9.0) for 15 min at 90°C, and then blocking for 1h with 5% normal goat serum or 1% BSA in TBS. Primary antibodies were diluted 1/200-1/500 with 0.1% BSA in TBS and stored overnight at 4°C prior to detection with secondary antibodies. The nuclei were counterstained with hematoxylin. For immunofluorescence staining, the nuclei were counterstained with DAPI.

### Meiotic chromosome-spread preparation and fluorescent immunostaining.
We obtained nuclear spreads by a method previously described. We obtained tissue sections as previously described.

### Antibodies.
Mouse polyclonal antibodies to Dmr7 were raised against a purified fusion protein containing the His-tag fused to the exon 4–9 recombinant protein of Dmr7. Other primary antibodies used in immunostaining were rabbit anti-cleaved caspase-3 (D175) (9661S, Cell Signaling, USA), rabbit anti-Scp3 (kindly provided by Dr. S. Chuma), goat anti-Scp3 (sc-20845, Santa Cruz, CA), rabbit anti-Scp1 (GT1/1/900, GenEx, USA), mouse anti-MLH1 (ab14206, Abcam, UK) and mouse anti-hH2AX (kindly provided by Dr. T. Takada). Secondary antibodies used were HRP-conjugated goat anti rabbit IgG (PI-1000, Vector Laboratories, Burlingame, CA), goat anti-rabbit Alexa 488 (A11008, Molecular Probes, USA), donkey anti-goat Alexa 594 (A11058, Molecular Probes, USA), and goat anti-mouse Alexa 594 (A21135; Molecular Probes, USA).

### Statistics.
The results of the experiments were expressed as the mean ± SEM ANOVA was used for statistical analysis of the results, and a p value of less than 0.05 was accepted as significantly different.

### Results
First we analyzed the expression of the Dmr7 protein of each stage of spermatogenesis and its localization in the spermatocytes using wild-type animals. Sections of testes, prepared from postnatal day (P) P7, P10, P14, P16, and P36 mice, were immunostained with α-Dmr7 antibody (Fig. 1A). No signals of Dmr7 protein were detected on Western blotting with the samples from testes of the P7 mice, while it was detected for the P10 testes (Fig. 1B). Germ cells differentiated to leptotene spermatocytes in the testes at the P10 stage, and our data suggest that expression of the Dmr7 gene reached a detectable level at that stage, while abundant amounts of Dmr7 transcript were observed at the pachytene stage in P14. Chromosome-protein spreading of mice spermatocytes with the α-Dmr7 antibody indicated that the Dmr7 protein was widely distributed in the nuclei, while condensed localization of Dmr7 was found in the XY body (Fig. 1C). The chromosomal structure changed dramatically at the pachytene stage, including synopsis of sister chromatids, and formation of the XY body, which was composed of X and Y chromosomes. Although reported transcriptional activity has been reported in the XY body (MSCI, meiotic sex chromo-
some inactivation), its function is not known.\(^{14-16}\) To understand in detail the localization of Dmrt7 protein in the nucleus, we carried out immuno double staining of the zygote and pachytene spermatocytes with \(\alpha\)Dmrt7 and anti-\(\gamma\)H2AX antibodies, and co-localization of both proteins at the XY body was confirmed (Fig. 1D). These results suggested that Dmrt7 was densely localized around the XY body in the pachytene spermatocytes. Since germ cells were observed at various stages in spermatogenesis depending on their position in the seminiferous tubules, particular stages in spermatogenesis were formed in the testicular sections independently of the location of the dissected seminiferous tubules, and their morphology allowed us to determine accurately the stage of spermatogenesis.\(^{17}\)

Immunostaining of testicular sections prepared from 6-month-old wild-type mice with the anti-Dmrt7 antibody indicated higher expression of it at the pachytene stage than at the diplotene stage (Fig. 1E). We assume that the remarkable expression of Dmrt7 starts during the late zygotene to the early pachytene stage, while it may decrease at the late pachytene stage and cease by the diplotene stage or the meiotic metaphase.

Previous papers have stated that spermatogenesis in Dmrt7 deficient mice was arrested, causing male infertility.\(^{8-10}\) We compared the morphology of 6-month-old testes from both genotypes by staining with hematoxylin and eosin. On the stained sections, vacuoles were formed with the loss of germ cells, and multinucleated cells indicated abnormal spermatogenesis (Fig. 2A). In addition, in the mutant seminiferous tubules, densely hematoxylin and eosin stained cells and cleaved caspase-3 positive apoptotic nuclei were observed (Fig. 2A, B). To determine accurately the stage at which spermatogenesis was arrested in the Dmrt7 mutant mice, we performed PAS staining of their testes, and we found no spermatogenesis at stage IV or later (Fig. 2C). Next, we determined the precise time
point during the pachytene stage in the mutant when spermatogenesis was arrested, and carried out RT-PCR for Cdc25c as a late-pachytene marker of testicular RNA and for HoxA4 as a mid-pachytene marker.18–20) HoxA4 expression was slightly decreased. In contrast, Cdc25c was not detected in the mutant testes (Fig. 2D). By PAS staining and RT-PCR, we observed that impaired spermatogenesis in the Dmrt7 deficient mice initiated at the mid-pachytene stage, as explained above, and then we determined whether the four subsequent events in meiotic prophase I were normal.

Synapsis, XY body formation, and MSCI, repression of retrotransposons, and meiotic recombination are essential for the completion of spermatogenesis.21–29) Impairment of any of these events would cause the arrest of spermatogenesis. To detect the synaptonemal complex, we immunostained chromosome spreads with αSCP3 antibody, and confirmed normal synapsis in the Dmrt7 deficient mice (Fig. 3A). Next, we counted the number of cells at each stage in meiotic prophase I, leptotene, zygotene, pachytene, and diplotene, and found increases in the numbers of leptotene spermatocytes, while we did not detect any cells at the diplotene stage (Fig. 3B). Hence we speculate that spermatogenic arrest in Dmrt7 deficient mice occurs between the late pachytene and the diplotene, because we found normal pachytene spermatocytes but no diplotene spermatocytes. Accumulation of histone variants and modification of histones or

Fig. 3. Normal Synapsis, XY Body Formation, and Abnormal MSCI in Dmrt7-Deficient Mice.
A, Analysis of XY body formation in wild type and Dmrt7 deficient mice by immunofluorescence staining. Surface-spread chromosomes were stained with 1/500 diluted anti-Scp3 (green) and 1/500 diluted anti-γH2AX (red) antibodies. Leptotene, zygotene, pachytene, and diplotene spermatocytes are shown. Scale bar = 10 μm. B, Distribution of spermatocytes at four different stages of meiotic prophase in wild-type and Dmrt7-deficient testes (P34). Meiotic prophase stages were detected by immunofluorescence staining with anti-Scp3 and anti-γH2AX antibodies. The numbers of spermatocyte at leptotene stage, the zygote stage, the pachytene stage, and diplotene stage in prophase I were counted individually. C, Expression analysis of mRNA from X- and Y-linked genes by quantitative RT-PCR in P17 wild-type and Dmrt7-deficient mice. Mecp2, Hprt, and Pgk1 are X-linked genes, while Ube1l and Rbmy1a1 are Y-linked genes, known to be targets of MSCI. Arhp is internal control. Error bars represent ± SEM. Asterisks indicate statistical significance as compared to the wild-type (p < 0.05). D, Expression analysis of mRNA from X- and Y-linked genes and their back-up genes in autosomes by quantitative RT-PCR in P17 wild-type and Dmrt7-deficient mice. Pgk1, Cem2, and Pdha1 are X-linked genes, known to be suppressed in MSCI, and Pgk2, Cem1, and Pdha2 are their back-up genes. Error bars represent ± SEM. Asterisks indicate statistical significance as compared to the wild type (p < 0.05).

Fig. 4. Overexpression of Retrotransposons and Failure of Complementation of Meiotic Recombination in Dmrt7-Deficient Mice.
A, Quantitative RT-PCR analysis of transcripts derived from transposable elements in testes from wild-type and Dmrt7-deficient mice (P17). Error bars represent ± SEM. Asterisks indicate statistical significance as compared to the wild-type (p < 0.05). B, Analysis of meiotic recombination by immunofluorescence staining in wild-type and Dmrt7-deficient mice. Testicular cell spreads at the pachytene stage were stained with 1/500 diluted antibody to Scp3 (green) and 1/200 diluted antibody to Mlh1 (red). Arrowheads indicate localization of Mlh1 on chromosomes. Scale bar = 10 μm.
transition of histones to XY body were reported as the XY body’s specific characteristic.\textsuperscript{30} $\gamma$H2AX is a well known protein that condenses in the XY body, and its localization is an index of XY body formation.\textsuperscript{23} We immunostained testicular paraffin sections and chromosome-spreads prepared from both genotypes with Sfp3 and $\gamma$H2AX antibodies. The paraffin section showed normal localization of $\gamma$H2AX at the XY body in the Dmrt7 deficient as well as the wild-type mice, and chromosomal spreads clearly showed distinct signals of $\gamma$H2AX on the XY body in the pachytene spermatocytes (Figs. 1D and 3A). Thus, the XY body was formed normally in the mutant testes (Fig. 3A). The impairment of MSCI observed in mice defective in certain genes\textsuperscript{23,24} is now understood to indicate that those genes are related to infertility. To determine whether MSCI was normal in the Dmrt7 deficient mice, we performed quantitative RT-PCR to evaluate the expression of several genes, Mecp2, Hprt, and Pgk1, on the X chromosome and Rbmyal1 and Ubel1 on the Y chromosome, which are regulated by MSCI. We found significant increase, (1.6–4.3 times), in the expression of all tested genes (Fig. 3C). Several genes on autosomes are activated during MSCI, complementing the function of essential genes on sex chromosomes that are silenced by MSCI (Fig. 3D). Quantitative RT-PCR in the Dmrt7 deficient mice indicated the expression of backup genes such as Pgk2, Pdha2, and Cetn1.\textsuperscript{31} Dmrt7 deficient mice showed no-upregulated expression of Pgk2, and Cetn1 at 50–60% of that of the wild-type mice. On the other hand, there were no significant differences between the expression of Pdha2 in the two types of mice (Fig. 3D).

Activation of transposable elements is known as one of the causal events leading to the arrest of spermatogenesis.\textsuperscript{25,26} In the testes of the Dmrt7 deficient mice, we analyzed the expression of LINE-1 and IAP, typical retrotransposons known to be suppressed during normal spermatogenesis, by quantitative RT-PCR (Fig. 4A). The expression of both LINE and IAP increased 2-fold. Immunostaining for chromosome spreads, prepared from Dmrt7 deficient and wild-type mice using an antibody against to Mlh1, a mismatch-repair protein, indicated the absence of expression of it in the mutant mice (Fig. 4B), suggesting that meiotic recombination became aberrant in the mutants.

**Discussion**

By immunohistochemistry, we observed the localization of the Dmrt7 protein in germ cells in wild-type testes in the first wave of spermatogenesis, and detected expression after P14 (Fig. 1A). On the other hand, we detected slight expression of Dmrt7 at P10. Given these results and the higher sensitivity of the Western blotting procedure, we estimated that the expression of Dmrt7 began at the P10 stage, and might have reached a stronger level after P14 (Fig. 1B). At P14, germ cells first differentiate to pachytene spermatocytes. We immunostained seminiferous tubules at the zygotene, pachytene, and diplotene stages and observed stronger signals of Dmrt7 in the pachytene spermatocytes, but no signals were detected in the zygotene or the diplotene spermatocytes (Fig. 1D, E). In addition, no immune-signal was detected in spermatogonia or spermatid (Fig. 1D, E). As for transcripts of the Dmrt7 gene, expression was detected in the testes at P10 and later stages, and it increased with sexual maturation. Thus, the Dmrt7 gene was activated after P10 and abundantly expressed in a spermatocyte-specific manner after P14 explained as above. We first clarified the expression profile of Dmrt7 following the progress of the spermatocyte.

In the testes of the Dmrt7 deficient mice, spermatogenesis was arrested with morphological impairments such as apoptosis at stage IV. This arrest was morphologically similar to the spermatogenic arrest caused by an unsuccessful pass through the pachytene checkpoint. In fact, no expression of the late pachytene marker gene was detected in the Dmrt7 deficient mice (Fig. 2D).

Our finding of impaired spermatogenesis in the Dmrt7 deficient mice strongly suggests that Dmrt7 have an essential role in pachytene spermatogenesis. Several events are specific to meiosis, including XY body formation, MSCI, the expression of piRNA, completion of meiotic recombination, and chiasma formation.\textsuperscript{33} We examined to determine whether these events occur normally in the Dmrt7 deficient mice. XY body formation, an essential event to overcome the pachytene checkpoint, was normal in Dmrt7 deficient mice (Fig. 3A). In those mice, immunostaining of Sfp3 and $\gamma$H2AX showed spermatocytes in different stages, as did those in the wild-type testes, but no diplotene spermatocytes were detected (Fig. 3B). Kim et al.\textsuperscript{31} reported that in Dmrt7 knockout mice, which they generated independently, a small number of diplotene spermatocytes can cross the pachytene checkpoint, and that these diplotene spermatocytes showed XY body degradation.\textsuperscript{5} Although, to date no one can delineate the precise function of Dmrt7 in XY body formation, these findings suggest that the essential role of Dmrt7 in the XY body. MSCI, an important event in spermatogenesis accompanied with XY body formation, was incomplete in the Dmrt7 deficient mice as compared with the wild-type mice (Fig. 3C). With the progress of MSCI, the expression of certain genes is known to be compensated for the expression of similar genes (backup genes) in the autosome.\textsuperscript{31} In the Dmrt7 deficient mice, the expression of Pgk2 and Cetn1 was significantly suppressed, while the expression of Pdha2 was as high as that in the wild-type mice (Fig. 3D). These results strongly suggest that Dmrt7 has important roles, especially in MSCI, during successive events in spermatogenesis, XY body formation, MSCI, and the upregulation of the expression of backup genes. Our results and the abnormal XY body in the diplotene reported by Kim et al.\textsuperscript{5} further suggest that Dmrt7 is related to the maintenance of the XY body, but not to XY body formation, followed by abnormal MSCI, which can occur during the process of impairment of XY body. Next we focused on a possible increase in the expression of the retrotransposons as the cause of the spermatogenesis arrest.

Recent reports of piRNA and DNA methylation have described a close relation between the activation of retrotransposon and spermatogenesis arrest.\textsuperscript{25,32,33} Xu et al.\textsuperscript{34} reported a 1.6-fold increase in the expression of LINE-1 in mice when they deleted the gene coding for specific piRNA.\textsuperscript{34} We suspect that the increase in the expression of retrotransposons aborted spermatogenesis, but the increase was about 2-fold (Fig. 4A), lower than...
those reported for other genes, whose knockout caused overexpression of retrotransposons and spermatogenesis arrest. piRNA synthesis comprises primary and secondary processing. In primary processing, RNAs are transcribed from genes of the piRNA cluster that originate in various retrotransposons. In primary processing, the primary products are transcribed from nuclear retrotransposons in the piRNA cluster region. Then piRNAs are generated and amplified in the cytoplasm by a mechanism known as the ping-pong cycle. Dmrt7, which is localized in the cell nucleus, might have a role in the transcription of piRNA. Meiotic recombination is an essential mechanism in the repair of damaged DNA, and disruption of it can cause spermatogenic arrest in the pachytene stage. Mutant mice deficient in such genes as Dmc1, responsible for meiotic recombination, showed infertile phenotypes.

In Dmrt7 deficient mice, Kim et al. confirmed the expression of Rad51, which is essential in the early stage of meiotic recombination, and they assumed that normal meiotic recombination occurred in the mutant mice. Although we checked the expression of Mlh1, which was functional in the late stage of meiotic recombination and is essential for mismatch repair, we did not detect its expression in the spermatocytes of the mutant mice immunologically (Fig. 4B). This suggest two hypotheses, i) Dmrt7 directly controls meiotic recombination, or ii) incomplete MSCI is directly induced by a lack of Dmrt7, followed by indirectly caused abnormality in meiotic recombination.

The results of this study of the mechanism of spermatogenesis arrest in Dmrt7 deficient mice might help to clarify the function of Dmrt7.

Acknowledgment

We are particularly grateful to Dr. Yuki Takada and Dr. Haruhiko Koseki (RIKEN Research Center for Allergy and Immunology) for advice and help in the meiotic chromosome spread procedure. We thank Dr. Tatsuyuki Takada (Ritsumeikan University) for anti-γH2AX antibody and Dr. Shinichiro Chuma (Kyoto University) for the anti-Scp3 antibody. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas, Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References