Disruption of Th2a and Th2b genes causes defects in spermatogenesis

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ABSTRACT

The variant histones TH2A and TH2B are abundant in the testis, but their roles in spermatogenesis remain elusive. Here, we show that male mutant mice lacking both Th2a and Th2b genes were sterile, with few sperm in the epididymis. In the mutant testis, the lack of TH2B was compensated for by overexpression of H2B, whereas overexpression of H2A was not observed, indicating a decrease in the total histone level. Mutant mice exhibited two defects: incomplete release of cohesin at interkinesis after meiosis I and histone replacement during spermiogenesis. In the mutant testis, secondary spermatocytes at interkinesis accumulated and cohesin was not released normally, suggesting that the retained cohesion of sister chromatids delayed the subsequent entry into meiosis II. In addition, impaired chromatin incorporation of TNP2 and degenerated spermatids were observed in the mutant testis. These results suggest that a loss of TH2A and TH2B function in chromatin dynamics or a decrease in the total histone levels causes defects in both cohesin release and histone replacement during spermatogenesis.

KEY WORDS: Cohesin, Histone replacement, Histone variants, Spermatogenesis, Mouse

INTRODUCTION

Two histone variants, TH2A and TH2B, are highly expressed in the testis (Trostle-Weige et al., 1982; Shires et al., 1976). Th2a and Th2b genes (Hist1h2aa and Hist1h2ba – Mouse Genome Informatics) localize adjacent on chromosome 17, and their expression is controlled by a shared promoter that is located between them (Huh et al., 1991), suggesting that TH2A and TH2B function together. Biochemical data suggest that TH2A and TH2B induce nucleosome instability (Li et al., 2005; Rao and Rao, 1987).

Recently, we have demonstrated high expression of TH2A and TH2B in oocytes and fertilized eggs, with a gradual decrease in their levels as zygotes differentiate into blastocysts (Shinagawa et al., 2014). Female mutant mice lacking both TH2A and TH2B develop normally and exhibit normal oocyte development. However, the number of pups from TH2A/TH2B-deficient females is ∼40% of that of wild type. TH2A and TH2B are maternal-effect proteins, and they contribute to the activation of the paternal genome after fertilization.

During spermatogenesis, primary spermatocytes progress through the following stages: leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase I and metaphase II (Hecht, 1998). Chromosomes begin to condense at leptotene, line up to form the homologous pairs that undergo recombination at zygotene and complete synopsis at pachytene. At metaphase I and II, homologous chromosomes and sister chromatids, respectively, are separated. Cohesin has an essential role in the connection of sister chromosomes. The mitotic cohesion complex consists of four subunits: Smc1α, Smc3, Rad21 and SA1/SA2 (Nasmyth, 2011). The meiotic cohesion complex contains several specific subunits, such as Rec8 and Rad21L (Herrán et al., 2011; Ishiguro et al., 2011; Lee and Hirano, 2011). During the metaphase I to anaphase I transition, cohesin complexes are cleaved by separase, which allows homologous chromosomes to segregate. At this stage, the cohesin complexes are retained at the centromere; during meiosis II, the cohesin complexes are cleaved, resulting in chromatid separation (Lee et al., 2008; Llano et al., 2008). During spermiogenesis, chromatin in round spermatids condenses as most of the histones are replaced by transition proteins, and then by protamines. In this study, we show that disruption of Th2a and Th2b causes defects in spermatogenesis, particularly during the two steps that involve the normal release of cohesin between meiosis I and II, and histone replacement during spermiogenesis.

RESULTS AND DISCUSSION

Defects in spermatogenesis in Th2a−/− Th2b−/− mice

Male homozygous mutant mice lacking both Th2a and Th2b genes are sterile (Shinagawa et al., 2014). The testis and epididymis of 3-month-old mutant mice showed a 28% and 17% reduction in weight, respectively, compared with the controls (Fig. 1A). The epididymis of mutant mice contained few sperm (Fig. 1B). Spermatogonia, primary spermatocytes at meiotic prophase and spermatocytes at metaphase I were found in the mutant testes at frequencies similar to those in the wild-type testes (Fig. 1C), indicating that the proliferation of spermatogonia and the development of spermatocytes from meiotic prophase to metaphase I was not affected by the loss of Th2a and Th2b genes. Secondary spermatocytes at interkinetics (the interphase between meiosis I and II) were more abundant in the mutant testes than in the control (Fig. 1C), suggesting extended interkinetics in mutant mice.

The results of western blotting, which used samples that spanned the first wave of spermatogenesis, demonstrated that the levels of TH2A and TH2B increased approximately two-fold in the testis at 30 days post-partum (days PP) compared with those at 16 days PP. However, the levels of canonical histones H2A and H2B were similar at 16 and 30 days PP (Fig. 1D). Thus, the ratio of variant and canonical histones was higher in spermatids than that in spermatocytes.
Abnormal retention of cohesin on chromosomes at interkinesis in Th2a−/−Th2b−/− testes

The synaptonemal complex and cohesin are released from chromosomes before metaphase II. Immunostaining of spread cells indicated that Rec8, a meiosis-specific subunit of the cohesin complex, and SCP3, still remained and colocalized in a significant number of mutant spermatocytes, but not in wild-type spermatocytes at interkinesis (Fig. 2A; supplementary material Fig. S2). Furthermore, a significant number of mutant spermatids contained SCP3 and Rad21, a cohesin complex component, whereas SCP1 was absent in wild-type and mutant cells (Fig. 2B,C). The cells with a 2C DNA content from the mutant testis, but not from the wild-type testis, contained Rad21 signals (Fig. 2D), suggesting that the increase in the DNA content was due to a retention of cohesin. Thus, SCP1 was released normally after the first meiotic division in mutant spermatocytes, but SCP3/cohesin was not, which may have delayed the subsequent entry of these cells into meiosis II. The cleavage of the cohesin subunit, Rec8, by separase is the primary mechanism that removes cohesin from the chromosome, although other mechanisms may also be involved (Nasmyth, 2011; Shintomi and Hirano, 2010). The loss of Th2a and Th2b genes may have induced a conformational change in chromosome-bound cohesin, which resulted in insufficient cleavage by separase.

Defects in the deposition of TNP2 on chromatin in Th2a−/−Th2b−/− spermatids

Morphologically normal spermatids were rare in mutant mice, and many degenerated step 10-16 spermatids, which showed an abnormal nuclear morphology, were observed (supplementary material Fig. S3A). These results suggest that disruption of Th2a and Th2b causes abnormalities in the development of spermatids into spermatozoa. The number of CENP-B foci was similar between wild-type and mutant secondary spermatocytes and spermatids (supplementary material Fig. S3B), suggesting that homologous chromosomes and sister chromatids had separated normally. During spermiogenesis, secondary spermatocytes at interkinesis. The separation of cells with 2C and 2′ DNA content may have been altered by PI affinity, possibly caused by the loss of TH2A/TH2B, because the binding of PI can be affected by differences in chromatin structure (Bender et al., 1997). The number of cells in fraction 8 with a 1C DNA content was lower in the mutant testis than in the control, suggesting that the spermatid population decreased. The increase in the population of MSY2-negative cells may have been due to an increase in the number of spermatogonia, resulting from defects in differentiation.

The synaptonemal complex is required for the formation of the synapsis and the joining of two homologous chromosomes, which each consist of two sister chromatids, in pachytene cells. This complex consists of three major components: the transverse filament containing SCP1, the lateral element containing SCP3 and cohesin, and the central element (Page and Hawley, 2003). Cohesin proteins in the arms of the sister chromatids are responsible for the cohesion of the two sister chromatids, whereas the transverse filaments connect to the lateral elements, thereby enabling them to merge four chromatids together. SCP3 immunostaining signals localized normally to pachytene chromosomes in wild-type and mutant testes (supplementary material Fig. S2), suggesting that synapsis formation was normal in cells of mutant mice. Consistent with this, histone γH2AX signals, which localize to the unpaired regions of chromosomes (Fernandez-Capetillo et al., 2003), in pachytene spermatocytes were found only on the single locus: the XY body (supplementary material Fig. S2).

Accumulation of spermatocytes at interkinesis in Th2a−/−Th2b−/− testes

The DNA content of spermatocytes was examined using fluorescence activated cell sorting (FACS) after staining spermatocytes with propidium iodide (PI) and an anti-MSY2 antibody, which distinguished spermatocytes and spermatids from spermatogonia and somatic cells. The results obtained using cells spanning the first wave of spermatogenesis indicated that the cells in fractions 4-7 were spermatocytes and the cells in fraction 8 were spermatids (supplementary material Fig. S1). The number of cells in fraction 7 with a 2C′ DNA content was higher in the mutant testis than in the control. This may have been due to an increase in the population of spermatogonia during the first wave of spermatogenesis. Whole testis cell extracts More than 45 stage XII tubules were counted per mouse. (D) Level of variant interkinesis spermatocytes; I-like, interkinesis spermatocyte-like; St9, step-9 leptotene spermatocyte; M, metaphase spermatocyte; R, round spermatid; Int, interkinesis spermatocytes; L, leptotene spermatocyte; M, metaphase spermatocyte; R, round spermatid; Int, interkinesis spermatocytes; I-like, interkinesis spermatocyte-like; St9, step-9 spermatid. Scale bars: 20 µm. The graph shows the percentage (mean±s.e.m.; n=3) of stage XII tubules harboring more than 10 interkinesis spermatocytes. More than 45 stage XII tubules were counted per mouse. (D) Level of variant histones during the first wave of spermatogenesis. Whole testis cell extracts (8 µg per lane) at indicated days post-partum were used for western blotting. Spg, spermatogonia; Spc, spermatocyte; Spt, spermatid.
Histones are replaced by transition nuclear proteins (TNP1 and TNP2), which are then replaced by protamines (Gaucher et al., 2010). The TNP2 immunostaining signal in mutant spermatids decreased substantially compared with that of the control (Fig. 3A,B), although the level of Tnp2 mRNA in mutant and wild-type testes was similar (supplementary material Fig. S3C). An abnormal nuclear morphology was evident in the mutant spermatids (Fig. 3B). The levels of TNP2 and protamine 2 in mutant spermatid extracts were lower than those in the controls (Fig. 3C). When spermatid whole extracts were separated into chromatin and non-chromatin fractions, TNP2 and protamine 2 were predominantly recovered in the chromatin fraction, and their levels in the mutant chromatin fraction were lower than those in the wild-type chromatin fraction, suggesting a defect in the chromatin incorporation of TNP2. Because there were no differences in the mRNA levels of several genes [Prm1, Foxj1, Ube2a, Rnf12 (Rlim) and Tpap (Papolb)] between wild-type and mutant spermatids with haploid genomes (supplementary material Fig. S3C), the defect in histone replacement may have been due to an abnormality in chromatin structure rather than to a decrease in haploid

Fig. 2. Cohesin is not properly released from the chromosome after meiosis I in mutant spermatocytes. (A) Representative images of Rec8 (green) and SCP3 (red) in interkinesis spermatocytes and elongating spermatids are shown. DNA was counterstained with TO-PRO-3 (blue). A higher magnification of the boxed regions show colocalization of Rec8 and SCP3 in mutant cells, but not in the control. Bar graphs show the average frequency of cells containing the Rec8 signal (n=3, ±s.e.m.). More than 40 nuclei were counted per mouse. (B,C) Testicular germ cells were immunostained with an anti-SCP1 (green) (B) or an anti-Rad21 (green) (C) antibody and an anti-SCP3 (red) antibody. DNA was counterstained with DAPI (blue). (D) Testicular germ cells with a 2C DNA content were separated by FACS and stained with an anti-Rad21 antibody (green) and PI (blue). The graph shows the percentage (mean±s.e.m.; n=3) of Rad21-positive nuclei. More than 30 nuclei were counted per mouse.

Fig. 3. Defects in histone replacement during spermiogenesis in Th2a−/− Th2b−/− mice. (A) Testis sections were immunostained with anti-TNP2 (green) and anti-γH2AX (red) antibodies. The stage of the seminiferous epithelial cycle was judged by the morphology of spermatids and the staining pattern of γH2AX. (B) Representative immunostaining images of spermatids are shown. The abnormal nuclear morphology is marked by blue triangles. (C) Spermatid lysates were separated by centrifugation into supernatant (non-chromatin) and pellet (chromatin) fractions, and were used for western blotting.
genome expression. In addition, we observed moderate differential expression in a few dozen genes in spermatocytes of mutant and wild-type testes (data not shown), suggesting that it was unlikely that the loss of Th2a and Th2b genes in spermatogonial stem cells caused these defects in spermatogenesis.

The results of western blot analysis of round and elongating spermatid extracts indicated that the H2A level was similar between the spermatids of wild-type and mutant mice, whereas the H2B level was higher in mutant spermatids than in the control spermatids (supplementary material Fig. S3D), suggesting the presence of a feedback mechanism. The results of histone liquid chromatography analysis showed that the H2B levels in mutant spermatocytes and spermatids were 2.4-fold and 1.7-fold higher than those in the wild-type spermatocytes and spermatids, respectively (Fig. 4; supplementary material Table S1). This is consistent with the report that TH2B levels in spermatocytes and spermatids are approximately twofold higher than the H2B level (Lu et al., 2009). However, the H2A levels were not significantly different in the mutant cells.

We speculate that the loss of TH2A/TH2B delays differentiation at interkinesis and blocks the replacement of histones during spermiogenesis. Although abnormal spermatids with a 4C DNA content are known to be generated by the inhibition of caspase-induced Rec8 cleavage (Kudo et al., 2009), incomplete release of cohesin in TH2A/TH2B-deficient spermatocytes did not result in the accumulation of abnormal spermatids with a 4C DNA content. Recently, Montellier et al. (2013) reported that mutant mice lacking TH2B expression exhibit no defect during spermiogenesis, indicating that a loss of TH2B alone is not sufficient to disturb histone replacement. Thus, loss of both TH2A and TH2B may be necessary to impair histone replacement and the normal release of cohesion at interkinesis. Alternatively, it is also possible that such defects are due to a decrease in the total histone level. In the mutant testis, the lack of
TH2B was compensated for by overexpression of H2B, whereas overexpression of H2A was not observed. Thus, the total amount of histones in the mutant cells was lower than that in the wild-type cells.

Multiple mechanisms may act in combination to destabilize the nucleosome and replace the histones. Hyperacetylation of canonical histones during spermatogenesis, especially H4, and multiple histone variants are thought to weaken DNA-histone octamers and nucleosome-nucleosome interactions (Govin et al., 2004; Govin et al., 2007; Gaucher et al., 2010). Nucleosomes containing hyperacetylated histones are thought to be more prone to displacement by protamines (Oliva et al., 1987). These previous reports are consistent with the observations that TH2A and TH2B, which induce an open chromatin structure (Shinagawa et al., 2014), are required for the replacement of histones by TNP-1. However, we cannot exclude the possibility that such defects are due to a decrease in the total histone level, because canonical histone H2A and H2B underdosage leads to defective meiosis in yeast (Hanlon et al., 2003).

Conclusions
We have demonstrated that loss of two genes encoding the histone variants TH2A and TH2B causes defects in spermatogenesis by disturbing cohesin release at interkinesis and histone replacement during spermiogenesis.

MATERIALS AND METHODS

Animals
TH2a/TH2b-deficient mice were generated as described previously (Shinagawa et al., 2014). Mutant mice were backcrossed with BALB/c mice at least five times before use in the experiments.

Histological and immunohistochemical examination of testes
Testes and caput epididymides were fixed in Bouin’s solution overnight, embedded in paraffin and sectioned. Sections were used for Hematoxylin and Eosin staining, periodic acid Schiff (PAS) staining and immunofluorescence. For immunostaining, sections were subjected to antigen retrieval and stained with the antibodies described in the methods in the supplementary material.

Separation of spermatids and western blot analysis
Round spermatids and elongating spermatids were isolated from a crude germ cell suspension from testes of two adult mice using the STAPUT method (Bellvé, 1993; Pivot-Pajot et al., 2003). Whole-cell extracts, and chromatin and non-chromatin fractions were used for western blotting. For further details, see methods in the supplementary material.

FACS analysis
A suspension of single germ cells was prepared as described by Bastos et al. (2005). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson). For further details, see methods in the supplementary material.

Immunostaining of testicular cells
For immunostaining of spermatocytes and spermatids, seminiferous tubules were spread as described by Peters et al. (1997), except that 80% ethanol was used for fixation. The antibodies used are described in the methods in the supplementary material.

qRT-PCR
Total RNA was extracted from spermatids and used for qRT-PCR. For further details, see methods in the supplementary material. Primers used are listed in Table S2 in the supplementary material.

Liquid chromatography mass spectrometry (LC-MS)
Histones were extracted from spermatocytes and spermatids by acid extraction and analyzed using LC-MS. For further details, see methods in the supplementary material.

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Competing interests
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Author contributions

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Supplementary material
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