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Affected Homologous Chromosome Pairing and Phosphorylation of Testis Specific Histone, H2AX, in Male Meiosis Under FKBp6 Deficiency

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Abstract. A gene for FK506 binding protein 6 (Fkbp6) expresses during a specific stage of male and female meiosis. Disruption of the gene influences male reproduction, i.e. arrests spermatogenesis, but not female reproduction. Using the mouse model (targeted disruption), the role of the gene in homologous chromosome pairing has been demonstrated in a previous study. For further understanding the function of Fkbp6 in chromosome synapsis, we evaluated chromosome pairings during male meiosis in the as/as rat, a spontaneous null mutation, and compared them with those of the mouse model. Electron microscopy of the pachytene nuclei unveiled several types of abnormal chromosome pairing in the rat model, as shown in the mouse previously. The frequencies of aberrant pairings in the knockout mice and mutant rats were 42 of 67 nuclei (62.7%) and 20 out of 74 nuclei (27.0%), respectively. In order to clarify the mechanism of male specific infertility in Fkbp6 deficiency, the localization of γH2AX, a marker protein of XY chromosome inactivation during male meiosis, was examined. Immunostaining of γH2AX unveiled normal localization of the molecule to XY chromosomes (XY body) in both models, showing the independency of Fkbp6 in sex chromosome inactivation. Besides the XY body, focal localization of γH2AX was observed in accordance with the unsynapsed chromosomes in both types of null animal. These results indicate the fundamental role of Fkbp6 in homologous chromosome synapsis during male meiosis. In conclusion, male specific infertility under Fkbp6 deficiency remains unsolved.

Key words: Chromosome pairing, Fkbp6 binding protein 6 (FKBp6), Meiosis, Mouse, Phosphorylated Histone H2AX (γH2AX), Rat

Meiosis is a germ cell specific type of cell division. One of the fundamental events in meiosis is homologous recombination, which enables exchange of maternal and paternal genes and generation of the resultant haploid gametocytes with genetic variation. The synaptonemal complex is a zipper-like protein structure that assembles along the paired homologous chromosomes during meiotic prophase to allow them to crossover and homologously recombine. To date, large numbers of molecules have been found to compose the synaptonemal complex. In many cases, impairment of the molecules induces aberrant chromosome pairing, resulting in meiotic arrest during the pachytene stage and exclusion of the cells by apoptosis.

FKBP6 is a protein that contains a prolyl isomerase/FK506 binding domain and tetratrico peptide protein-protein interaction domains/TPR motifs [1]. Deficiency of Fkbp6 causes spermatogenic arrest during meiotic prophase in mice and rats [2–4]. The protein has been shown to express during pachynema and to localize in the synaptonemal complex. Despite expression of the gene in both the male and female pachynema, non-homologous pairing or failure of synapsis has been detected in male, but not female, deficient mice [4]. This male specific disorder during meiosis raised the question of whether Fkbp6 has a role in male specific events during meiosis.

In the pachytene stage, the XY body (sex body) is formed in the nucleus region surrounding sex chromosomes [5]. The XY body is thought to function in transcriptional silencing of sex chromosomes, as evidenced by decreased uridine incorporation into XY body [6] or sex body exclusive localization of RNA polymerase II [7, 8]. Recent studies have indicated that a testis specific histone, histone H2AX, is a possible key molecule for histone remodeling of the XY body. Phosphorylated H2AX (γH2AX) has been suggested to be an inaccessible form of histone for transcriptional enzymes that functions to decrease expressions of X and Y-linked genes during meiosis [9, 10]. In addition, the phosphorylated molecule has been detected in the unpaired region of non-homologous pairing, which is proposed to have a role in activation of the meiotic recombination checkpoint [11].

For further understanding the role of Fkbp6 in homologous synapsis, we evaluated chromosome pairings in deficient rats and compared the incidence of aberrant pairing with that of knockout mice. Because of our interest in the male specific disorder, we also investigated the distribution of γH2AX, a marker molecule of sex chromosome inactivation, in the pachytene nuclei of both mutant animals.

Materials and Methods

Animals and preparation of germ cells

All the protocols for use of animals were approved by the Ani-
mal Care Committee of the National Institute of Agrobiological Sciences. Testes from adult Fkbp6 deficient mice (targeting inactivation, Fkbp6 +/-; provided by Dr. JM Penninger) and an Fkbp6 deficient rat (spontaneous mutation, as/as; raised at the National Institute of Agrobiological Sciences) and a phenotypically normal heterozygous mouse and rat (Fkbp6 +/- and +/-as, respectively) were used. Testicular cells were collected mechanically, and the cells were processed by the surface spread method [12]. A sufficient number of cells was obtained from the testes of single Fkbp6 +/-, +/-as and as/as males and from two to three Fkbp6 -/- males.

For silver staining, hypotonically treated cells with 0.5% NaCl were spread on plastic coated glass slides and then fixed with 4% paraformaldehyde (PFA) and 0.03% SDS solution. For immunostaining, hypotonically treated cells were spread on glass slides and then fixed with 2% PFA and 0.03% SDS solution. The slides were stored at –20 C until immunostaining.

Electron microscopy
Surface spread cells on plastic coated glass slides were stained by the one-step AgNO3 staining method [13]. Briefly, cells were developed in colloidal developer [mixture of 33.3% (w/v) AgNO3 solution and 2% gelatin and 1% formic acid solution; 2:1] at 70 C. When sufficiently colored (normally 2–3 min), cells were rinsed in water and then dried. The plastic film with the cells was removed from the glass slide and put on a grid. Silver-stained nuclei were observed randomly under an electron microscope (JEM-1010; JOEL, Tokyo, Japan). More than 60 pachytene nuclei were examined for each genotype of mouse and rat.

Immunostaining
Immunostaining of the surface spread cells was performed in accordance with Dresser et al. [14]. Briefly, the preparation was incubated with antigen dilution buffer (ADB; PBS with 2% BSA and 0.05% Triton-X 100) at 37 C and was then reacted with antibodies at 37 C. An anti-SCP3 rabbit IgG (gifted by Drs. N Nakatsuji and S Chuma) and a γH2AX monoclonal antibody (Upstate, Lake Placid, NY, USA) were used at a dilution of 1:1,000 and 1:200, respectively. Subsequently, an anti-rabbit donkey IgG conjugated with Rhodamine (Chemicon, Temecula, CA, USA) and an anti-mouse goat IgG conjugated with Alexa Fluor® 488 (Molecular Probes, Eugene, OR, USA) were applied, and then immunostained nuclei were examined under a laser scanning microscope (FV300; Olympus, Tokyo, Japan). More than 150 germ cell nuclei for each type of mouse and over 200 germ cell nuclei for each type of rat were examined, respectively.

Results

Chromosome pairing in FKBP6 deficiency
In Fkbp6 +/- mice, all the pachytene nuclei examined displayed normal pairing. Homologous autosomes paired completely at the early pachytene stage, and the pairing lasted till the end of the stage. In comparison with the synchronous change of autosomes, sex chromosomes displayed a distinguishable form of assembly with their thickened and partially aligned shapes in the restrictive region, the pseudoautosomal region. Assembly and disassembly of XY pair was in accordance with a previous report [14]. Maximum formation of XY synapsis during the early stage (Fig.1A) was followed by progressive desynapsis as the pachytene stage proceeded. At the late pachytene stage, end-to-end attachment of X and Y chromosomes was observed. In the Fkbp6 +/- mice, various types of abnormal pairing were detected, including partial synapsis, asyn-
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apsis and non-homologous synapsis (Fig.1B and C). Non-homologous pairing between sex chromosomes and autosomes was also observed, which was in accordance with Crackower et al. [4].

Some of the pachytene nuclei of the +/- rat displayed partial synapsis in autosomes (3 out of 68 nuclei) such that normal homologous pairing occupied 95.6% of the pachytene nuclei examined. Sex chromosomes showed the rat-specific change as reported by Joseph and Chandley [15], i.e. being thickened in the early pachytene, assembly in the pseudoautosomal region and subsequent disassembly in the middle and late pachytene stages. The Fkbp6 deficiency caused abnormality in chromosome pairing in the rat. Similar to the affected mouse nuclei, asynapsis or partial synapsis of homologous pairs and non-homologous association between the asynaptic parts of the chromosome were detected. Interestingly, complete autosynapsis of autosomes and sex chromo-

Fig. 3. Localization of γH2AX in the pachytene nuclei of the Fkbp6 deficient (-/- and as/as) and control (+/- and +/as) males. A and B: mice. C and D: rats. Chromosome pairing was visualized by SCP3 staining. Control pachytene nuclei (A and C) exhibited localization of γH2AX in the restricted region surrounding XY (XY body). In the deficient nuclei (B and D), γH2AX localization corresponding to the affected pairings was observed. (arrows). In D, the diffusive localization of γH2AX (arrowhead) seems to include XY.

Fig. 4. Frequency of aberrant localization of γH2AX in the Fkbp6 deficient (-/- and as/as) and control (+/- and +/as) pachytene nuclei. Immunostained pachytene nuclei (n>100) were categorized into five groups according to the pattern of γH2AX staining: restricted localization of γH2AX in the XY body (solid), XY body and accumulation at the region of autosomal abnormal pairing (horizontally hatched), diffusive localization surrounding the XY body (vertically hatched), accumulation in regions of abnormally paired autosomes and diffusive localization surrounding the XY body (diagonally hatched) and no staining (open).

somess was seen in some nuclei (Fig. 1E and F).

When the incidence of abnormal pairing were compared between the knockout mouse and the mutant rat, lower incidence were detected in the rat than in the mouse (Fig. 2). In the mouse model, approximately 63% (42 out of 67 pachytene nuclei) showed disorder of chromosome pairing, and 27 out of 42 affected nuclei displayed abnormal sex chromosome pairing. This frequency was in accordance with those observed by Kolas et al. [16]. In contrast, 20 out of 74 pachytene nuclei (27.0%) were affected in the rat model, and 12 out of 20 were affected in terms of XY pairing.

Localization of γH2AX in pachytene nuclei

The surface spread cells were reacted with SCP3 antibody, which acted as an indicator of chromosome pairing. Assembly of the axial elements of autosomes during the zygotene stage results in appearance of aligned thick SCP3 elements in Fkbp6 +/- mice and +/+as rats. In the pachytene stage, X and Y chromosomes could be distinguished from autosomes. Their axial elements were also stained with the SCP3 antibody, and it was determined that the pseudoautosomal region restricted synapsis, as observed by electron microscopy. The entire distribution of γH2AX molecule in the nucleus was observed from leptotene to early zygotene. As the zygotene stage proceeded, however, the distribution became restricted, only where synapsis has not been completed yet. When fully synapsed (in pachynema), γH2AX was detected in only the region surrounding the XY chromosome, i.e. XY body (Fig. 3A and C). The XY body associated γH2AX localization persisted till the nuclei entered the diplotene stage.

The appearances of SCP3 and γH2AX in the leptotene and zygotene nuclei of the Fkbp6 +/- and as/as males were indistinguishable from those in +/- mice and +/+as rats. Some, but not all, pachytene
nuclei, however, exhibited aberrant morphology of the SCP3 axis, such as multi-combined or partially assembled thin axis. In many cases, these aberrant parts accompanied γH2AX distribution other than the XY associated pattern of γH2AX (Fig. 3B and D). We analyzed the incidence of aberrant γH2AX distribution (Fig. 4). In the knockout mice, approximately 25.0% of the cells exhibited localization of the phosphorylated molecule associated with abnormal chromosome pairing in addition to the XY body, whereas 3.1% of nuclei lacked the molecule. In the mutant rats, localization of γH2AX relevant to abnormal chromosome pairing was detected in approximately 14.0% of nuclei, whereas only one out of 214 nuclei (0.9%) exhibited no localization of the molecule. In some cases, γH2AX was diffusively localized in the region corresponding to the XY pair (Fig. 3D), which seems to reflect non-homologous pairing between sex chromosomes and autosomes.

Discussion

The present results evidence an essential role of FKBP6 in rat homologous chromosome pairing during spermatogenesis. Pairing occurs under the function of the synaptonemal complex, which allows recombination of paternal and maternal genomes. The synaptonemal complex is a highly conserved protein machinery consisting of three major components, the transverse filament and lateral and central elements [17]. Expression of Fkbp6 in the testis has been reported not only in rodents [4, 18], but also in humans [1]. Although the role of FKBP6 in the synaptonemal complex is unclear, a biochemical association with SCP1, a major component of the transverse filament, has been proposed in mice [4]. Taking this into account, FKBP6 could be a critical molecule in homologous pairing during male meiosis.

The as homozygous rat exhibited a lower incidence of abnormal pairing than the Fkbp6 null mouse, which suggests a species specific difference in the influence of the gene deficiency. This might be caused by insufficient, but not abolished, expression of the protein in the mutant rat testis. In the Fkbp6 +/- mouse, both mRNA and protein expressions were completely repressed in the testis. In the mutant rat testis, however, decreased expression of mutated mRNA, which reflected a partial deletion of genomic DNA, including exon 8, has been demonstrated [4]. We have also detected a lower molecular form of protein in the mutant rat testis than in the wild type by Western blotting using antibodies generated with an N-terminus antigen and with recombinant full length FKBP6 (data not shown). The mutant type protein might function to some extent in homologous pairing.

There is no doubt that aberrant chromosome pairing at the pachytene stage triggers apoptosis to eliminate germ cells. Incidence of abnormal pairing under Fkbp6 deficiency was observed in part of the spermatocyte (63 and 27% in mice and rats, respectively), despite complete arrest of spermatogenesis in both animals. The mechanisms that determine the fate of germ cells with normal pairings are unclear, but it is noteworthy that Fkbp6 deficiency causes an additional abnormal phenotype, i.e., aggregation of ribosome in the cytoplasm of the pachytene spermatocyte and dysfunction of the blood testis barrier [19]. The influenced distribution of ribosomes in the cytoplasm might reduce protein synthesis and result in degeneration of the cells. The blood testis barrier is significant in creation of a favorable environment for germ cells to differentiate. Male sterility due to Osp/Claudin11 deficiency, which exhibits incomplete formation of the testicular tight junction between Sertoli cells, evidences the importance of barrier function in spermatogenesis [20]. Taken together, the male sterility due to Fkbp6 deficiency should be understood as the combined consequence of abnormal events, i.e., aberrant chromosome pairings, ribosome aggregation at the pachytene stage and dysfunction of the blood-testis barrier.

The H2AX phosphorylation of XY chromosomes during meiotic prophase is considered to be essential for XY inactivation during male meiosis [9, 21]. ATR (a PI3K-like protein kinase; a candidate kinase for H2AX phosphorylation) has been demonstrated to localize along the unsynapsed part of the XY chromosome in association with BRCA1 [10]. The present study revealed the XY preferential localization of γH2AX in most of the pachytene nuclei of both Fkbp6 deficient animals (75% in the mouse and 96.9% in the rat). These results may suggest an independency of Fkbp6 from this testis specific histone remodeling system. On the other hand, another type of remodeling system including histone H3 methylation and H4 acetylation has been proposed to play roles in XY inactivation [22]. The role of this chromatin modification system in FKBP6 deficiency should be determined in order to clarify the relationship between this male specific disorder during meiosis under Fkbp6 deficiency and XY chromosome inactivation.

The accumulation of γH2AX to the autosomal unsynapsed region in Fkbp6 deficiency was in accordance with the observations of Perera et al. [11]. They suggested that the accumulation was caused by ATR that colocalized with TopBP1. Accumulation of γH2AX in the unsynapsed autosomal region has been observed in conjunction with delayed pairing, abnormal pairing in conjunction with impaired expression of critical genes during meiosis and quadrivalents by translocation [10, 23–25]. We also observed diffusive distribution of γH2AX related to the XY body, which seemed to reflect abnormal pairing including the XY body. This observation was comparable to the accumulation of γH2AX in t(X;16) and t(Y;15) quadrivalents [24, 25]. Transcriptional silencing of the unpaired chromosome region has been demonstrated, and the resultant impairment of crucial genes might activate a meiotic checkpoint to eliminate the cells by apoptosis [24]. This hypothesis is consistent with the increased apoptosis in Fkbp6 deficiency [4].

In conclusion, we have determined the essential role of Fkbp6 in homologous chromosome pairing during male meiosis. In order to elucidate male specific disorders in reproduction under the null mutation, further investigation of XY inactivation as well as extensive study of female reproduction in mutants should be undertaken.

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