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INVITED REVIEW

Reproductive Biology

The molecular control of meiotic double-strand break (DSB) formation and its significance in human infertility

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Meiosis is an essential step in gametogenesis which is the key process in sexually reproducing organisms as meiotic aberrations may result in infertility. In meiosis, programmed DNA double-strand break (DSB) formation is one of the fundamental processes that are essential for maintaining homolog interactions and correcting segregation of chromosomes. Although the number and distribution of meiotic DSBs are tightly regulated, still abnormalities in DSB formation are known to cause meiotic arrest and infertility. This review is a detailed account of molecular bases of meiotic DSB formation, its evolutionary conservation, and variations in different species. We further reviewed the mutations of DSB formation genes in association with human infertility and also proposed the future directions and strategies about the study of meiotic DSB formation.

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INTRODUCTION

Production of normal haploid gametes through meiosis is a highly conserved and critical process associated with the fertility in sexually reproducing species.[1] Errors impeding the meiotic progression always lead to aneuploidy and/or sterility.[2] Meiosis is a special cell division process which contains two rounds of sequential chromosome segregation, preceded by a single round of DNA replication. Meiosis I is responsible for choreographic arrangement of homologous chromosomes and their subsequent accurate segregation, while sister chromatids segregate in meiosis II which is generally considered as a reduction phase.[3] During prophase I, each pair of homologous chromosomes is joined by at least one crossover to achieve high fidelity of segregation.[4] The formation of these crossovers requires the production of meiotic DNA double-strand breaks (DSBs) that are ultimately repaired during homologous recombination.[5] Generally, around 200–300 programmed meiotic DSBs are produced during leptotene to zygotene stages in mice, which are sufficient for chromosomes to search for their homologous partner.[6] It is also obvious that an increase in the number of DSBs will increase the risk of mutations, chromosome rearrangement, and genomic instability. On the other hand, insufficient DSB formation may cause homologous pairing defects, inaccurate chromosomal segregation, and decreased genetic diversity in subsequent generations.[7] In addition, normal distribution of meiotic DSBs protects functional genomic components such as promoters and enhancers from being affected.[8] Thus, the timing and frequency of DSB formation and their location must be strictly regulated.[7]

THE DETERMINATION OF HOTSPOTS BY PRDM9

Meiotic DSBs are not randomly introduced in the genome but mainly occur at discrete regions called hotspots.[6] In mammals, meiotic DSB formation is mostly regulated by the sequence-specific DNA-binding PR domain-containing protein 9 (PRDM9). PRDM9 is a meiosis-specific histone H3 methyltransferase and a major determinant of meiosis recombination hotspots in mammals.[9,10] It specifically expresses in reproductive system, from the preleptotene to pachytene stage.[11] PRDM9 has three functional domains: an N-terminal Krüppel-associated box (KRAB)-related domain that promotes protein binding and transcriptional inhibition, a central PRDI-BF1 and RIZ1 homology (PR/SET) domain which has histone methyltransferase activity, and a C-terminal C2H2-type zinc finger domain that determines the specificity for DNA recognition.[12] PRDM9 recognizes specific DNA sequences through C2H2-type zinc fingers and catalyzes lysine 4 of histone H3 (H3K4) and lysine 36 of histone H3 (H3K36) trimethylation *in vivo* through its PR/SET domain.[12]

Different genetic methodologies have been used to investigate the *in vivo* function of PRDM9, and its targeted deletion in mice caused meiotic arrest and sterility in both sexes.[11] Ectopic DSB formation and limited synapsis were observed in *Prdm9*-knockout mice due to defects of DSB repair. In *Prdm9*-knockout mice, though 99% of the

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DSB hotspots were altered, 94% of the hotspots were still located on trimethylation of histone H3 lysine 4 (H3K4me3) sites.[8] Thus, it is tempting to speculate that H3K4me3 has an active role in controlling the DSB formation. In wild-type mice, H3K4me3 sites were not only enriched in DSB hotspots, but they also marked the gene promoters, enhancers, and possibly other functional genetic elements that were actively transcribed. In the absence of PRDM9, DSBs will be initiated at PRDM9-independent H3K4me3 sites, and an increased number of DSB hotspots at transcription start sites will be generated,[8] indicating that PRDM9 regulates the distribution of DSBs and also protects key functional genomic components.

Intriguingly, although most recombinant hotspots in the genome are PRDM9-dependent, hotspots on the pseudoautosomal region (PAR) are PRDM9-independent in mice, suggesting that DSB formation in the PAR could be different from that of autosomes.[8] Hence, there might be other mechanisms to determine DSB sites on the PAR, which is worthy to be explored.

FROM H3K4ME3 TO DSBS: THE REGULATION OF HOTSPOTS

The number of hotspots marked by PRDM9 exceeds the number of DSBs in meiocytes; thus, DSB sites need to be "picked out" from these hotspots. The initiation of programmed DSBs occurs at the chromosome axes and is controlled by various factors that define the recombination events.[13] Thus, PRDM9-mediated recombination hotspots must be brought to the chromosome axes under the action of certain proteins before the initiation of DSBs, which further provides a suitable and spatial environment for subsequent recombination events to induce DSB formation (**Figure 1**).

The KRAB domain of PRDM9 plays a decisive role in tethering and recruiting other proteins to form multi-protein complexes on hotspot regions of the chromosome axes.[14,15] Four proteins, namely CXXC domain-containing 1 (CXXC1), Ewing sarcoma 1 (EWSR1), euchromatic histone methyltransferase 2 (EHMT2), and chromodomain-containing Y chromosome-like (CDYL), can directly interact with KRAB domain.[15] In addition, PRDM9 also interacts with cohesion proteins of meiotic recombination protein REC8 homolog (REC8) and cohesin subunit SA-3 (STAG3), as well as the synaptonemal complex protein 1 (SYCP1) and synaptonemal complex protein 3 (SYCP3).[15,16] Parvanov *et al*.[15] believed that during early leptotene, some PRDM9 molecules in the nucleus bind to the recombination hotspots and form dimers, with one of the subunits binding to DNA. Both subunits are involved in the methylation of histone H3K4 and H3K36, ensuring the accumulation of H3K4me3 and trimethylation of histone H3 lysine 36 (H3K36me3) on PRDM9.[17] In combination with CDYL and EHMT2, the KRAB domain of PRDM9 restricts the level of trimethylation on 2–4 nucleosomes on each side.[17,18] At the end of leptotene, CDYL and EHMT2 are removed from the complex, and the recombination hotspots are bounded with PRDM9, while the neighboring nucleosomes bind with EWSR1 and CXXC1. These complexes can interact with REC8 and SYCP3, thus transferring the recombination hotspots within their own chromosomal loops to the chromosome axes.[15]

Recent studies have demonstrated that CXXC1 deficiency does not affect the formation of H3K4me3 and DSBs but impede the repair of DSBs in spermatocytes, suggesting that CXXC1 is a downstream factor of PRDM9 rather than a protein to assist PRDM9 recruitment.[19,20] EWSR1 is also involved in DSB repair by facilitating the formation and resolution of Holliday junction during pachytene.[15] Deletion of *Ewsr1* in mice does not affect the expression and localization of PRDM9 and its related interacting proteins; however, the levels of H3K4me3 and H3K36me3 at the recombinant hotspots were drastically reduced at leptonema and zygonema. *Ewsr1*-deficient spermatocytes can still form the PRDM9-dependent DSB hotspots, but the DSB activity and distribution were altered, which in turn disturbed the number and distribution of crossovers. In addition, EWSR1 interacts with phosphorylated REC8 to assist the interaction of PRDM9 and cohesion complex in chromosome axes, suggesting that EWSR1 participates in the selection of H3K4me3-activated hotspots to form DSBs. Interestingly, the functions of EWSR1 in meiotic cells, including the promotion of PRDM9-dependent H3K4/K36 trimethylation and the connection between PRDM9 and phosphorylated REC8, are interdependent. Thus, the attachment of PRDM9 to chromosome axes may stabilize the entire complex and eventually allow efficient H3K4/K36 trimethylation.[21]

THE ASSEMBLY OF DSB FORMATION MACHINERY ON CHROMOSOME AXES

To ensure the efficient DSB formation after the attachment of PRDM9, DSB formation machinery should be assembled on the chromosome axes. During mammalian meiosis, DSB formation machinery proteins, including interactor of HORMA domain-containing protein 1 (HORMAD1) protein 1 (IHO1), meiotic recombination protein REC114 (REC114), and meiosis-specific protein MEI4 (MEI4), are all located on the axes and observed from preleptotene to pachytene stages.[22–25] Knockout mice of these genes are all sterile due to spermatogenic arrest with no postmeiotic cells in the seminiferous tubules. The spermatocytes from these mutant mice failed to recruit DSB repair proteins, including replication protein A complex (RPA), meiotic recombination protein DMC1/LIM15 homolog (DMC1), and DNA repair protein RAD51 homolog 1 (RAD51), indicating the failure of DSB formation.[22–25]

Yeast-two hybrid has provided the evidence that the recruitment of IHO1 on the axes is assisted by HORMAD1.[25] As HORMAD1 is only localized on unsynapsed axes, it was suggested that DSB formation is regulated by synapsis through the disassembly of HORMAD1.[26] REC114 and MEI4 are then reinvigorated through IHO1 to form IHO1-REC114-MEI4 complex.[25] Finally, with the help of PRDM9, they will activate meiotic recombination protein SPO11 (SPO11) to generate DSB on hotspots (**Figure 1**).[25]

Recently, a new member of the DSB formation machinery, ankyrin repeat domain-containing protein 31 (ANKRD31), was identified.[27,28] Similar to DSB formation machinery proteins, ANKRD31 is also expressed in preleptotene to early pachytene spermatocytes and demonstrates co-localization and interaction with IHO1 and REC114. Interestingly, unlike other DSB formation machinery proteins, the deletion of *Ankrd31* in mice does not block the formation of meiotic DSBs, but its absence changes the DSB distribution in a PRDM9-independent pattern,[27,28] suggesting that ANKRD31 may play a role in mediating the connection between PRDM9 and DSB formation machinery (**Figure 1**).

THE GENERATION OF DSBS BY SPO11 COMPLEX

It is well known that meiotic DSBs are generated by topoisomerase VI (TopoVI), which is consisted of two subunits: SPO11 and type 2 DNA topoisomerase VI subunit B-like (TOPOVIBL; **Figure 1**).[29,30] TopoVI belongs to the type IIB family of topoisomerases, whose activity can facilitate the unwinding of supercoiled DNA and induce DNA decatenation through cleavage and ligation cycles.[31,32] SPO11 is widely conserved in eukaryotes and is an ortholog of subunit A

of TopoVI DNA topoisomerase (TopoVIA).[33] TOPOVIBL has a structural similarity with the B subunit of TopoVI topoisomerase and is also highly conserved from yeast to mammals.[30] The interaction between TOPOVIBL and SPO11 suggested that they can catalyze the formation of DSBs as a complex and the phenotypes of *Spo11*- and *Top6bl*-knockout mice are fundamentally the same: infertility in both sexes due to the lack of meiotic DSBs.[30,34,35]

In humans and mice, there are two major SPO11 variants, SPO11α and SPO11β.[36] The functions of both isomers are different, with SPO11β inducing DSBs on autosomes from leptotene to early zygotene, while SPO11α catalyzing DSBs in the PAR of X and Y chromosomes at late zygotene stage.[37] Moreover, *Spo11α*-knockout mice still display SPO11β-mediated global DSB formation; however, the pairing and synapsis of X and Y chromosomes are impaired due to the lack of DSBs in PAR, suggesting that SPO11α plays a role in the formation of DSBs on PAR.[37] The difference between sex chromosomes and autosomes on DSB formation in spermatocytes might be due to the marked morphological differences of X and Y chromosomes. In spermatocytes, pairing and recombination must take place between sex chromosomes to ensure their segregation during anaphase I. To ensure this, at least one DSB should be initiated and repaired to form crossover on the small PAR region between X and Y chromosomes.[37] It should be noted that there is only one DSB foci per 10 Mb on average in mice, whereas the PAR is only about 700 kb. Studies have shown that PAR chromatin forms shorter loops and longer axes compared to autosomes, which facilitates DSB formation at higher frequencies.[37] A recent study showed that due to the presence of mo-2 sequence in PAR, RMMAI proteins (REC114, MEI4, Meiosis inhibitor protein 1 [MEI1], ANKRD31, and IHO1) could be recruited to PAR directly.[38] In addition, ultrastructural analysis of PAR showed that in late zygotene, the sister chromatids could be split apart and re-condensed, then normal pairing and synapsis take place during the transition of zygotene to pachytene stage.[38] Therefore, due to its particularity, sex chromosomes have different structures and regulatory modes for meiotic DSB formation.

The number of meiotic DSBs is highly regulated and can be assessed directly by counting the foci of recombinases RAD51 and DMC1.[6] However, the results are affected by the efficiency of recombinase recruitment and DSB repair, which make it unreliable under certain conditions. Thus, the detection of SPO11-oligo, a by-product associated with DSB formation, is used to study the level and distribution of DSB formation.[39–42] This method was also used to prove the regulatory role of serine-protein kinase ATM (ATM) in DSB formation.[40] As a key kinase in DNA damage response, ATM is important for efficient DSB repair[43] and the loss of ATM in spermatocytes leads to a large-scale change on DSB distribution.[40] In particular, large Mb-scale regions that are typically DSB-poor (cold) regions appear to be more sensitive to ATM loss than DSB-rich (hot) regions. In addition, in wild-type spermatocytes, the SPO11-oligo density of XY chromosomes is much lower than that of autosomes, but in *Atm*-knockout mice, the SPO11-oligo density of these regions is higher than the genomic average.[44] Thus, in meiotic cells, ATM may be activated by DSBs, triggering a negative feedback loop that inhibits toxic numbers of DSBs.[45]

Lukaszewicz *et al*.[44] proposed an ATM-mediated DSB formation model. In detail, DSBs preferentially form at hotspots in the chromatin loops that attached to axes. In response to DSBs, ATM is activated and phosphorylates REC114, HORMAD1, or other proteins. Through inhibiting the formation or disrupting the stability of the IHO1-REC114-MEI4 complex, phosphorylation events can inhibit additional DSB formation on the same or adjacent chromatin loops. Similarly, phosphorylation of HORMAD1 near DSB sites prevents the assembly of the IHO1-REC114-MEI4 complex, resulting in DSB formation at other nearby chromatin loops.[44]

THE EVOLUTION OF DSB FORMATION IN DIFFERENT ORGANISMS

DSB formation is a highly conserved process, although different mechanisms for DSB regulation are observed among different species (**Table 1**).[5] DSB formation has been vigorously studied in *Saccharomyces cerevisiae*, and three complexes have been reported so far. The first complex composed of Spo11 and SKI complex subunit tryptophan-aspartic acid (WD) repeat protein SKI8 (Ski8). Ski8, a WD repeat protein, interacts with the C-terminal of Spo11.[46] In *Schizosaccharomyces pombe*, their orthologs, Rec12 (Spo11) and Rec14 (Ski8), are also proved to interact with each other.[47] It has been demonstrated that Ski8 is evolutionarily conserved and is necessary for DSB formation in *S. cerevisiae* and *S. pombe*,[48] but the exact mechanism underlying DSB formation in these organisms is still not clear. On the other hand, three Spo11 paralogs have been identified in *Arabidopsis thaliana*, two (spo11-1 and spo11-2) of which are required for DSB formation, while the function of spo11-3 is dispensable.[49,50] Interestingly, Ski8 was not crucial for meiotic DSB formation in *A. thaliana*, raising the question of its functional conservation for meiosis outside fungi.[51] However, it is not clear whether the ortholog of mammalian Ski8, WDR61, plays a role in meiotic DSB formation.

Another complex that has been identified is consisted of meiotic recombination protein REC102 (Rec102) and meiotic recombination protein REC104 (Rec104) in *S. cerevisiae*. These two proteins interact with each other, and Rec104 also interacts with Spo11. Rec102 and Rec104 bind to chromatin to facilitate Spo11 dimerization, DNA binding, and effective nuclear retention.[52] In *S. pombe*, meiotic recombination protein rec6 (Rec6) is considered as another important partner for meiotic DSB formation, and it forms complex with two other proteins, meiotic recombination protein rec12 (Rec12) and meiotic recombination protein rec14 (Rec14).[53] It is important to mention that Rec6 does not interact with Rec12 or Rec14 individually in yeast two-hybrid assays but shows interaction with them when they were co-expressed.[54]

In addition, *S. cerevisiae* Rec102-Rec104 complex may form a bridge between Spo11 and a third complex Rec114-Mei4-Mer2, which is analogous to REC114-MEI4-IHO1 in mammals.[46] It has been reported that Rec114-Mei4-Mer2 complex is required for DSB formation, and Mer2 colocalizes with Rec114 and Mei4 at chromosomal axis sites.[55,56] In *S. pombe*, meiotic recombination proteins rec24, rec7, and rec15 (Rec24, Rec7, and Rec15) were detected on both axes and DSB sites and also form a complex, in which Rec7 directly interacts with Rec24.[57] As the recruitment of Rec15 to DSB sites depends on Rec24 and Rec7 rather than Rec12, suggesting that the Rec7-Rec24-Rec15 complex may recognize some specific features of DSB sites. In addition, Mei4-dependent protein 2 (Mde2) protein (lacking *S. cerevisiae* ortholog) is critical for DSB formation and interacts with Rec15 to stabilize the Rec7-Rec24-Rec15 complex. Interestingly, it also binds to Rec14, and thereby connecting the two sub-complexes.[54] In *A. thaliana*, DSB formation also requires *A. thaliana* Mei4 ortholog multipolar spindle 1 (PRD2).[58] However, *A. thaliana* Rec114 orthologs dual specificity protein phosphatase PHS1 (PHS1) is not required for DSB formation but functions in DSB repair and homologous pairing.[59] Other factors that are required for DSB formation such as MEI1 and its *A. thaliana* ortholog putative recombination initiation defect 1 (PRD1) have been found using genetic methods. They encode proteins that contain armadillo repeat sequence similar to importins, but their activities are not known.[60–63]

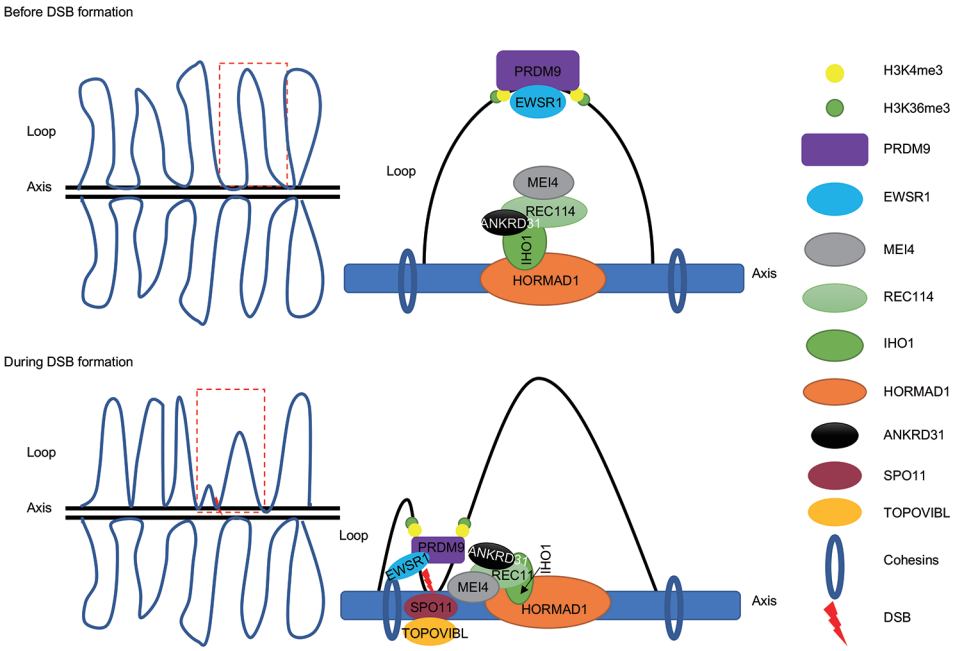

Figure 1: Model of DSB formation. In early meiotic prophase, chromosomes are organized in a classical loop axis structure. PRDM9 binds to the chromatin loops and determines the meiotic hotspots (only one sister chromatid is presented in the figure). PRDM9 can modify the conformation of surrounding nucleosomes by catalyzing H3K4me3 and H3K36me3 deposition. EWSR1 can interact with phosphorylated REC8 (pREC8) to facilitate the interaction of PRDM9 and cohesion complex on the chromosome axis. With the assistant of HORMAD1, DSB formation machinery proteins, such as IHO1, REC114 and MEI4, are anchored on the axis. Then, the complex of SPO11 and TOPOVIBL may be recruited at the PRDM9-binding sites and catalyze DSBs. ANKRD31, which we know little now, may play a role in mediating the connection between PRDM9 and DSB formation machinery. ANKRD31: ankyrin repeat domain-containing protein 31; DSB: double-strand break; EWSR1: Ewing sarcoma 1; H3K4me3: trimethylation of histone H3 lysine 4; H3K36me3: trimethylation of histone H3 lysine 36; HORMAD1: HORMA domain-containing protein 1; IHO1: interactor of HORMAD1 protein 1; MEI4: meiosis-specific protein MEI4; PRDM9: PR domain-containing protein 9; REC114: meiotic recombination protein REC114; SPO11: meiotic recombination protein SPO11; TOPOVIBL: type 2 DNA topoisomerase VI subunit B-like.

Table 1: Double-strand break formation-related genes in different organisms

Saccharomyces cerevisiae	*Schizosaccharomyces pombe*	*Arabidopsis thaliana*	*Caenorhabditis elegans*	*Mus musculus*
SPO11	*rec12*	*SPO11-1*	*spo-11*	*Spo11*
		SPO11-2		
		SPO11-3[b]		
SKI8	*rec14*	*SKI8*[b]	–	–
REC102	*rec6*	*MTOPVIB*	-	*Top6bl*
REC104	–	–	-	–
REC114	*rec7*	*PHS1*[b]	-	*Rec114*
MEI4	*rec24*	*PRD2*	-	*Mei4*
MER2	*rec15*	–	-	*Iho1*
–	–	–	-	*Ankrd31*
–	–	*PRD1*	-	*Mei1*
HOP1	*hop1*	*ASY1*[b]	*htp-3*	*Hormad1*
				Hormad2
–	*rec25*	–	–	–
–	*rec27*	–	–	–
TEL1	*tel1*[a]	*ATM*[b]	*atm-1*[a]	*Atm*
–	–	–	–	*Prdm9*
SPP1	*spp1*[a]	*SPP1*[a]	*cfp-1*[a]	*Cxxc1*[b]
–	–	–	–	*Ewsr1*

[a]Unvalidated in meiotic DSB formation; [b]dispensible for DSB formation. DSB: double-strand break; –: no homolog has been reported.

Meiosis-specific protein HOP1 (Hop1) is another meiosis-specific protein that is required during DSB formation and promotes inter-homolog rather than inter-sister recombination during DSB repair in *S. cerevisiae*.[64] It contains a Hop1p, Rev7p, and MAd2 (HORMA) domain that is thought to recognize chromatin condition and is considered to be an adapter to recruit other proteins. In prophase of meiosis I, Hop1 is located along the meiosis chromosome axes and its enrichment corresponds to the high-frequency DSB regions. SPO11 accessory proteins Rec114/Mei4/Mer2 are also enriched in these regions, and in the absence of *Hop1*, Mer2 would not be recruited on the chromosome and these events are also conserved in *S. pombe*.[65,66]

In mouse, two Hop1 orthologs, HORMAD1 and HORMAD2, have been identified.[26] HORMAD1 locates at the unsynapsed chromosome axes and is disassembled on synapsed axes. HORMAD1 directly interacts with IHO1 and is responsible for its recruitment to the unsynapsed axes, indicating a regulatory mechanism of HORMAD1 to initiate meiotic DSBs on unsynapsed chromosomes.[25] However, despite the loss of IHO1 foci from chromosome axes, *Hormad1*-deficient spermatocytes still displayed meiotic DSB formation at a lower level, suggesting that the recruitment of IHO1 to chromosome axes is important but not essential for meiotic DSB formation.[25] In *Caenorhabditis elegans*, several HORMAD-like proteins which considered as structural components of the meiosis axes have been identified. In particular, HORMA domain-containing protein (HTP-3) is associated with the chromosome axes during prophase I and is necessary for DSB formation. Interestingly, HTP-3 interacts with double-strand break repair protein mre-11 (MRE-11) to provide a potential link between DSB formation and repair.[67–69] In contrast, *A. thaliana* meiosis-specific protein ASY1 (ASY1) has limited homology to *S. cerevisiae* Hop1, and it seems not to be necessary for DSB formation

because lacking asy1 in *A. thaliana* did not result in any obvious changes in DSB formation.[70]

In mammals, PRDM9 and its related proteins connect the site of DSB to the DSB formation machinery; however, in *S. cerevisiae*, it is mediated by COMPASS component SPP1 (Spp1), a member of the complex of proteins associated with Set1 (COMPASS) chromatin modification complex.[71] Through the plant homeodomain (PHD) finger domain, Spp1 interacts with the axis-associated protein Mer2.[72] In *S. pombe*, meiotic recombination proteins rec25 and rec27 (Rec25 and Rec27) are two linear elements without ortholog identified in *S. cerevisiae*, and they are necessary for DSB formation.[73] Rec25 and Rec27 seem to recognize some unknown features of DSB sites. In addition, Mde2, Rec15, and Rec24 interact with DSB catalytic unit, which is also associated with the DSB site in the absence of Rec12.[54] The analysis of genome-wide DSB mapping in *S. pombe* shows that H3K4me3 does not play an important role in DSB formation, and unlike *S. cerevisiae*, the DSB-axis interaction may not require Spp1.

MUTATIONS IN DSB FORMATION GENE AND MALE INFERTILITY

Meiotic prophase I defects (such as errors that impede meiotic recombination) invariably result in germ cell developmental arrest, and it is one of the important causes of azoospermia, but still the detailed mechanism is unknown. At present, a few cases of male infertility caused by mutations in genes that are required for DSB formation have been reported, and these mutations generally manifested nonobstructive azoospermia (NOA) phenotype. For example, two known pathogenic mutations in *MEI1* have been reported: *MEI1* c.3307C>T in a consanguineous Tunisian family[74] and MEI1 c.[1196+1G>A];[2209del] compound heterozygous mutations in an NOA patient.[75] In addition, a missense mutation, c.556G>A, in *SPO11* was found in a consanguineous family with NOA phenotype.[76] However, none of these mutations had been functionally verified, and they were screened as pathogenic mutations just because their knockout mice were infertile (**Table 2**).

DSB FORMATION GENE MUTATION AND FEMALE INFERTILITY

Mutations in DSB formation-related genes can lead to developmental disorders and abnormalities in follicular formation. Developmental disorders caused by such mutations can lead to embryo arrest, hydatidiform mole, or miscarriage.[75,77] By performing whole-exome sequencing on 65 women with recurrent hydatidiform moles, mutations in different DSB formation-related proteins were found in several cases, including *TOP6BL* c.783dup insert mutation, *TOP6BL* c.1501T>C missense mutation, *REC114* c.334-1G>A splice site mutation, *MEI1* c.3452G>A missense mutation, and *MEI1* c.[1196+1G>A];[2209del] compound heterozygous mutation.[75] The investigation that oocytes were capable of fertilization and 5% of them produced androgenetic zygotes in *Mei1*-deficient female mice may provide an explanation for the formation of hydatidiform mole or miscarriage in women. In addition, another study identified *REC114* c.397T>G missense mutation in a consanguineous family and further found a rare *REC114* c.546+5G>A splice site mutation in 579 women with *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) failure due to abnormal fertilization or early embryo arrest. *In vitro* experiments showed that *REC114* c.397T>G missense mutation decreased the level of REC114 protein and affected the stability of MEI4, while *REC114* c.546+5G>A splice site mutation affected the alternative splicing of REC114 (**Table 2**).[77]

FUTURE EXPECTATION

DSB formation is the initiation step for meiotic recombination and the basis of homologous chromosome pairing and synapsis. In mammals, DSB formation sites are determined by PRDM9, which induces the formation of nucleosomal-depleted region through H3K4me3 and H3K36me3. PRDM9-bond chromosomes are recruited from the chromosome loops to the chromosome axes by DSB formation machinery via proteins that interact with its KRAB region. After the determination of hotspot region, programmed DSBs are catalyzed by the evolutionarily conserved proteins, SPO11 and TOPOVIBL. ATM participates in regulating the number and distribution of DSB through negative feedback that maintains the number of DSBs in a specific range and prevents the abnormal distribution of DSBs. This is the current general understanding of meiotic DSB formation, but there are still a lot of issues that need further scholarly investigation.

1. Which hotspots will be selected by PRDM9 to form DSBs? It is known that the hotspots of H3K4me3 and PRDM9 are more than the final number of DSBs. It is interesting to know how these DSB sites are "picked out" from numerous H3K4me3 site to form DSBs
2. Which proteins mediate the transfer of PRDM9 from chromosome loops to axes? CXXC1, the ortholog of Spp1 in yeast, does not affect DSB formation in mammals, while another protein ESWR1, which interacts with the KRAB domain of PRDM9, helps PRDM9 to bind to phosphorylated REC8. Thus, more proteins that are involved in this process need to be identified
3. The location of MEI4 on the axes is MEI1 dependent, while IHO1-REC114-MEI4 is the component of DSB formation machinery. Interestingly, PRD1, the ortholog of MEI1 in *A. thaliana*, interacts with spo11-1. Therefore, it is necessary to study whether MEI1 is

Table 2: Double-strand break formation gene-related mutants in human infertility

Gene	*cDNA variant*	*Protein change*	*Mutation type*	*Clinical presentation*	*Reference*	*KO mouse phenotype*	*Reference*
SPO11	c.556G>A	p.Glu186Lys	Missense	NOA	76	Meiotic arrest	34, 35
TOPOVIBL	c.783dup	p.Glu262*	Insertion	One miscarriage and two HMs	75	Meiotic arrest	30
	c.1501T>C	p.Ser501Pro	Missense	RHMs	75		
REC114	c.334-1G>A	–	Splicing	One miscarriage and three CHMs	75	Meiotic arrest	24
	c.397T>G	p.C133G	Missense	Abnormal fertilization, embryo arrest	77		
	c.546+5G>A	–	Splicing	Abnormal fertilization, embryo arrest	77		
MEI1	c.1196+1G>A	–	Splicing	Male: NOA Female: six miscarriages and one CHM	75	Meiotic arrest	61
	c.2209del	p.Val736Ser fs*31	Frameshift				
	c.3452G>A	p.Trp1151*	Nonsense	Four miscarriages and four HMs	75		
	c.C3307T	p.R1103W	Missense	NOA	74		

DSB: double-strand break; NOA: nonobstructive azoospermia; HMs: hydatifiform moles; RHMs: recurrent HMs; CHMs: complete HMs; KO: knockout; *SPO11*: Meiotic recombination protein SPO11; *TOPOVIBL*: type 2 DNA topoisomerase VI subunit B-like; *REC114*: meiotic recombination protein REC114; *MEI1*: *Mus musculus* meiosis inhibitor protein 1; –: unknown

the protein that connects DSB formation machinery to SPO11

4. The mechanism that regulates the number and distribution of DSBs by ATM is still need to be discovered. For example, in *Atm*-knockout mice, DSB levels increased 10-fold as compared to wild-type and the length of SPO11-oligo became longer. Thus, due to kinase function of ATM and axial distribution of DSBs, it would be interesting to identify the axial proteins that are phosphorylated by ATM in a DSB-dependent manner
5. DSB formation in sex chromosomes undertakes a special mechanism. Acquaviva *et al*.[38] suggested that before the production of DSBs in sex chromosomes, their sister chromatid axes should split apart and collect large amounts of RMMAI proteins (REC114, MEI4, MEI1, ANKRD31, and IHO1). Thus, the mechanism to recruit these proteins for DSB formation in sex chromosomes and the dynamics of their sister chromatid separation need to be investigated in the future
6. Till now, only ten mutations in four genes related to DSB formation (*SPO11*, *TOP6BL*, *REC114*, and *MEI1*) have been reported to be associated with human infertility.[74–77] Obviously, DSB formation-related genes are far more than these four and some genes such as *IHO1* and *ANKRD31*[25,27,28] are recently discovered in mammals; hence, more genetic mutations of DSB formation-related genes are remained to be discovered. In addition, some reports conducted polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) on specific DSB formation genes in infertile patients and found some single nucleotide polymorphism (SNP) sites that may cause human infertility.[78,79] However, this method only targets specific genes; mutations from other genes cannot be excluded. Besides the known mutations in DSB formation, mutations leading to abnormal DSB distribution have not yet been detected and such mutations may be the next emerging issue that needs to be explored. For example, mutations in *PRDM9*, *ANKRD31*, and *EWSR1* may cause abnormal DSB distribution and meiotic failure. Hence, further understanding of meiotic DSB formation will contribute to the clinical diagnosis and treatment of human infertility.

AUTHOR CONTRIBUTIONS

XHJ and QHS planned the project and supervised the overall work. YL and YFW wrote the manuscript. HWJ, RK, QQH, FI, XHJ, and QHS were involved in the article modification. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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