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An intact Pms2 ATPase domain is not essential for male fertility

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Abstract

The DNA mismatch repair (MMR) machinery in mammals plays critical roles in both mutation avoidance and spermatogenesis. Meiotic analysis of knockout mice of two different MMR genes, *Mlh1* and *Mlh3*, revealed both male and female infertility associated with a defect in meiotic crossing over. In contrast, another MMR gene knockout, *Pms2* (*Pms2^{ko/ko}*), which contained a deletion of a portion of the ATPase domain, produced animals that were male sterile but female fertile. However, the meiotic phenotype of *Pms2^{ko/ko}* males was less clear-cut than for *Mlh1*- or *Mlh3*-deficient meiosis. More recently, we generated a different *Pms2* mutant allele (*Pms2^{cre}*), which results in deletion of the same portion of the ATPase domain. Surprisingly, *Pms2^{cre/cre}* male mice were completely fertile, suggesting that the ATPase domain of *Pms2* is not required for male fertility. To explore the difference in male fertility, we examined the *Pms2* RNA and found that alternative splicing of the *Pms2^{cre}* allele results in a predicted *Pms2* containing the C-terminus, which contains the *Mlh1*-interaction domain, a possible candidate for stabilizing *Mlh1* levels. To study further the basis of male fertility, we examined *Mlh1* levels in testes and found that whereas *Pms2* loss in *Pms2^{ko/ko}* mice results in severely reduced levels of *Mlh1* expression in the testes, *Mlh1* levels in *Pms2^{cre/cre}* testes were reduced to a lesser extent. Thus, we propose that a primary function of *Pms2* during spermatogenesis is to stabilize *Mlh1* levels prior to its critical crossing over function with *Mlh3*.

Keywords

Pms2; *Mlh1* stability; male infertility

1. Introduction

The DNA mismatch repair (MMR) machinery in mammals carries out critical roles in both mutation avoidance and meiosis. Evidence for these dual roles came from early studies of various MMR knockout mice, which showed, in addition to the expected phenotypes of

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increased spontaneous mutation and cancer [1–3], the unanticipated male and/or female infertility [4–10]. Analyses of *Mlh1* and *Mlh3* gene knockout mice revealed both male and female infertility associated with a defect consistent with the two proteins having a critical role in meiotic crossing over [5–7]. Other studies in mice and yeast indicate that Mlh1 and Mlh3 form a heterodimer critical for normal meiotic crossing over [11]. In addition, the Mlh3 protein possesses an endonuclease function that is critical for normal levels of reciprocal recombination between homologs [12], which in turn is absolutely essential for normal meiosis [13].

In contrast to the male and female infertility exhibited by both *Mlh1* and *Mlh3* knockout mice, *Pms2* knockout (*Pms2^{ko}*) mice were male sterile, but female fertile. Analysis of *Pms2* null spermatogenesis showed a greatly reduced number of sperm of abnormal morphology [4]. However, the phenotype of *Pms2*-deficient male meiosis was less interpretable than was the case for *Mlh1*-deficient meiosis, which clearly showed a greatly increased number of univalent chromosomes in anaphase, characteristic of a failure of crossing over [5]. In contrast, the phenotype of *Pms2^{ko}* male meiosis was relatively subtle, suggesting a possible defect in chromosome pairing during prophase of male meiosis [4]. Here, based on unexpected findings with a mouse model intended to facilitate stochastic alteration of target genes, we show that the *Pms2* ATPase domain is not necessary for normal meiosis. Based on initial results, we suggest that *Pms2* has an important role in stabilizing Mlh1 during spermatogenesis.

2. Materials and Methods

2.1 Mice and DNA

Mice were housed in a specific pathogen free HEPA filtered room and were fed a diet of LabDiet PicoLab Rodent Diet 20. All experiments were approved by the IACUC committee at OHSU. *Pms2^{ko}* [4] and *Pms2^{cre}* [14] mice were generated and genotyped as previously described. *Mlh1* [5], *Mlh3* [7] and *Pms1* [2] knockout mice were generated and genotyped as previously described. Frozen sections of testes tissue were prepared as previously described [15]. Testis volume was calculated by estimating the radius determined with a dissecting scope and using the formula, $\text{volume} = 4/3 \times \pi \times r^3$. Sanger sequencing was performed at the OHSU DNA sequencing core using either PCR products or Topo TA (Invitrogen) cloned PCR products.

2.2 RNA analyses

RNA was extracted from fresh or frozen testes using the miRNeasy Mini Kit (Qiagen). cDNA was made from RNA using random hexamers and the SuperScript III First Strand Synthesis Kit (Invitrogen). Quantitative PCR for *Pms2* levels was performed using 0.5X Sybr Green (Invitrogen) in a Rotor-Gene Q (Qiagen). Expression level of genes neighboring *Pms2* was calculated from band intensity with Adobe Photoshop. Primers used are listed in Supplemental Table 1.

2.3 Protein analyses

Each sample tested by Western represented a single testis or thymus extracted from individual mice of the relevant genotypes. Total protein was extracted from fresh or frozen tissue with *Brij 150 Lysis buffer* (0.01M Tris, 2mM EDTA, 0.15M NaCl, 1% Brij 97, 0.1% NP40, 25 nM Leupeptin, 25 nM Aprotinin, 25 nM AEBSF). Samples were then treated as previously described [16]. Briefly, samples were heated in SDS sample buffer at 95 °C for 5 min, and 20–50 µg total protein run on an 8% SDS-PAGE gel. The protein was transferred to a PVDF membrane and probed simultaneously with mouse anti-human MLH1 (Cat #: 51–1327GR), PMS2 (Cat #: 556415), and MSH6 (Cat #: 610918) antibodies (BD Biosciences) at dilutions of 1:1000, 1:500, and 1:2500, respectively. Membranes were then probed with goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch) at a dilution of 1:1000. The secondary antibody was visualized using enhanced chemiluminescence (Perkin Elmer). Mlh1 signal within each lane was normalized to the Msh6 signal using Adobe Photoshop. Multiple, independent Westerns were performed with each blot containing at least one wild-type (WT) sample in addition to experimental samples for normalization across the different genotypes and blots. Student t-test was used to determine significance between Mlh1 levels in the *Pms2^{ko}* and *Pms2^{cre}* testes.

3. Results

3.1 Male fertility despite lack of an intact Pms2 ATPase motif

As reported previously, we developed a mouse model designed to facilitate stochastic activation of Cre recombinase by targeting an out-of-reading frame *cre* sequence to the *Pms2* locus, thus creating the *Pms2^{cre}* allele [14]. The targeting strategy placed Cre expression under the control of the *Pms2* promoter and as for the original *Pms2^{ko}* allele [4], resulted in deletion of exon 2, which encodes a portion of a ATPase domain critical for mutation avoidance (Figure 1A). As expected from our analyses of *Pms2^{ko/ko}* mice [4], *Pms2^{cre/cre}* mice are strong mutators [15, 17]. To our great surprise, we found that *Pms2^{cre/cre}* male mice, in contrast to *Pms2^{ko/ko}* males, were fully fertile, producing normal size litters (Table 1) and exhibiting normal sperm morphology (Figure 1B). The fertility of *Pms2^{cre/cre}* male mice which lack an intact ATPase domain indicates that the ATPase of Pms2 is dispensable for normal spermatogenesis.

3.2 Alternative splicing in Pms2^{cre} testis

We first examined RNA expression levels of *Pms2* and closely linked genes to determine if transgene insertion of the targeting cassette resulted in aberrant regulation. We found no differences between *Pms2^{+/+}*, *Pms2^{cre/cre}*, and *Pms2^{ko/ko}* testes in the expression levels of *Pms2* (Figure 2A) or six neighboring genes (*Cyth3*, *Usp42*, *Eif2ak1*, *Ankrd61*, *Aimp2*, and *Rsph10b2*) (Figure 2B), suggesting that the differences in fertility between genotypes was not caused by changes in RNA expression due to transgene insertion. In addition, because loss of *Usp42* function results in male infertility [18], we sequenced the exons from *Usp42* in *Pms2^{ko}* mice and did not find any truncating or nonsense mutations.

Because *Pms2* exon 2 was deleted from the DNA, any changes in *Pms2* expression levels would nonetheless result in an out-of-frame Pms2 protein. However, alternative splicing of

the *Pms2* RNA for the *Pms2^{cre}* allele might result in a Pms2 polypeptide different from the *Pms2^{ko}* allele. Therefore, we compared Pms2 RNA species from the different alleles by amplifying *Pms2* cDNA from exon 1 to exon 5 and confirmed by sequencing (Figure 3). We obtained results consistent with *Pms2^{cre}* RNA being spliced differently than *Pms2^{ko}* RNA in the testes. As predicted, for both *Pms2^{cre}* and *Pms2^{ko}*, exon 2 containing a portion of the ATPase domain [4] was absent in both cases from the cDNA. As predicted, *Pms2^{ko}* RNA was spliced from exon 1 to exon 3, which results in an out-of-frame protein (Figure 2C). However, *Pms2^{cre}* RNA is spliced in two different ways: 1) From exon 1 to exon 4, which is out of frame, and 2) from exon 1 to exon 5, which places the remainder of the RNA in frame (Figure 2D). Therefore, a fraction of *Pms2^{cre}* RNA from testes should generate a Pms2 protein that contains an intact C-terminus. We analyzed further *Pms2^{cre}* testis tissue via Western analysis, using a C-terminal specific monoclonal antibody, but this failed to provide direct evidence of Pms2 C-terminus expression. However, Western analysis is not a particularly sensitive technique. The Pms2 protein has three known functional domains: ATPase, endonuclease and Mlh1 interaction domain, which based on our RNA splicing analysis is predicted to be expressed by *Pms2^{cre}* but not the *Pms2^{ko}* allele (Figure 2E). These results, in combination with previous results showing that male mice mutated in the Pms2-endonuclease motif were fertile [19], suggested to us that the MutL interaction domain is the critical domain for male fertility. Therefore, we examined the stability of Mlh1 following Pms2 loss in somatic (thymus) and germline (testis) tissues.

3.3 Loss of both Pms2 and Pms1 destabilizes Mlh1 in mouse thymus

Previous studies had shown that Pms2 and Mlh1 form the MutL α heterodimer via interaction between their C-termini [20–23]. Furthermore, loss of this interaction due to *Mlh1* mutation resulted in greatly reduced levels of Pms2 [20] strongly suggesting that formation of the heterodimer was necessary for Pms2 protein stability. However, cultured cells [1, 16] or thymus from *Pms2^{ko/ko}* mice had no detectable effect on the level of Mlh1 (Figure 4A). Mlh1 also forms heterodimers with Pms1 (MutL β) or Mlh3 (MutL γ) [24, 25]. To determine which Mlh1 partners are important for Mlh1 stability, we first examined thymus tissue from mice with *Pms1* or *Mlh3* gene knockouts and different gene knockout combinations with *Pms2*. In the thymus, only gene knockouts of both *Pms2* and *Pms1* resulted in destabilization of Mlh1, whereas *Mlh3* gene knockout had minimal effect on Mlh1 stability (Figure 4A). These results indicate that Pms2 in conjunction with Pms1 is critical for Mlh1 stabilization in the mouse thymus.

3.4 Loss of Pms2 alone is sufficient to destabilize Mlh1 in mouse testis

Because Mlh1 is critical for meiotic crossing over, we asked whether loss of Pms2 in the testes of *Pms2^{ko/ko}* mice would destabilize Mlh1. Western analysis of whole protein isolates revealed that Mlh1 levels were in fact decreased by ~70% in the testes of *Pms2^{ko/ko}* mice (Figure 4B), indicating a role for Pms2 in stabilizing Mlh1 in the mouse testis. Gene knockout of *Mlh3*, the key heterodimeric partner of Mlh1 for meiotic crossover, resulted in Mlh1 instability, but not to the same degree as the *Pms2* gene knockout (Figure 4B). Gene knockouts of both *Pms2* and *Mlh3* resulted in the lowest level of Mlh1. In contrast, *Pms1* gene knockout had a minimal effect on Mlh1 stability (Figure 4B). These results show the

importance of Pms2 in maintaining normal Mlh1 levels in the mouse testis and implicate the destabilization of Mlh1 as the basis for *Pms2*^{ko/ko} male infertility.

Because *Pms2*^{cre/cre} male mice are fertile, we next examined Mlh1 levels in the testes of *Pms2*^{cre/cre} mice and found that Mlh1 was reduced only 40–50% (Figure 4C), equating to a ~2-fold higher expression level than for *Pms2*^{ko/ko} mice, supporting increased stabilization of Mlh1. Comparing protein expression levels in whole testes between *Pms2*^{cre/cre} and *Pms2*^{ko/ko} mice is valid because, in contrast to *Mlh1*^{-/-} mice [6], *Pms2*^{ko/ko} testes, although exhibiting reduced spermatid numbers, were similar in overall size and contained similar numbers of spermatocytes and spermatogonia when compared to wild-type and *Pms2*^{cre/cre} mice (Figure 1C–D) [4]. Thus, we suggest that the higher Mlh1 levels in *Pms2*^{cre/cre} testes relative to *Pms2*^{ko/ko} accounts for the observed fertility. The apparent increased stabilization of Mlh1 levels in *Pms2*^{cre/cre} mice and alternative splicing of the *Pms2*^{cre} allele resulting in expression of the Mlh1-interacting domain, in contrast to the *Pms2*^{ko} allele [4], suggest that Pms2 is important for male fertility due to its role in the stabilization of Mlh1 during spermatogenesis. Further studies, for example the analysis of staged male germ cells are required to substantiate this hypothesis.

4. Discussion

Implications for the function of Pms2 in male meiosis can be made based on previous studies and the findings presented here. There are three known functional domains/motifs within mammalian Pms2, which are important for mutation avoidance: 1) The ATPase motif located in the N-terminal region of the protein [26–28], 2) the endonuclease motif located in the C-terminal region [29–31] and, 3) a domain in the C-terminus that is necessary for heterodimerization with Mlh1 [20–23]. Despite the absence of critical residues within the ATPase domain in *Pms2*^{cre/cre} male mice, these mice are fully fertile, indicating that the ATPase activity of Pms2 is not necessary for normal spermatogenesis. Edelmann and colleagues derived mice with a missense mutation predicted to disrupt function of the endonuclease motif located in the C-terminal region of Pms2 [19]. They reported that males homozygous for the mutation were strong mutators, but fertile, strongly suggesting that the endonuclease activity of Pms2, although necessary for mutation avoidance, was dispensable for spermatogenesis [19].

Our findings presented here suggest that the fertility of *Pms2*^{cre/cre} males is due to the production of a Pms2 polypeptide that contains the C-terminal Mlh1-interacting domain, which is not expressed in *Pms2*^{ko/ko} mice. Although modest, we propose that the resultant increase in Mlh1 protein in *Pms2*^{cre} testes compared to *Pms2*^{ko} testes, and presumably in spermatocytes, fosters adequate formation of the Mlh1/Mlh3 heterodimer, which based on studies in yeast [32], mice [33], and humans [34] is necessary for normal levels of crossing over and the resultant normal disjunction of homologs during first division meiosis [10]. These studies could be furthered by examining spermatocyte chromosome spreads from *Pms2*^{cre/cre} mice for Mlh1 foci, adding the alternative splice variant back to the *Pms2*^{ko} mice to rescue the male infertility, or by generating Mlh1 hypomorphic mice to determine if there is a threshold level of Mlh1 needed for spermatogenesis and gametogenesis. In addition, based on the fertility of *Pms2*^{ko/ko} females [4], and the findings presented here, we further

suggest that stabilization of Mlh1 protein levels by the presence Pms2 is not critical during oogenesis. In summary, our results indicate that the ATPase of Pms2 is dispensable for proper spermatogenesis and presumably meiosis. Further, based on our results and those of others mentioned above, we propose that the primary function of Pms2 protein in spermatogenesis and meiosis is to stabilize Mlh1 protein levels, thus ensuring adequate levels prior to formation of the cross-over and meiosis-critical Mlh1/Mlh3 heterodimer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A fully intact Pms2 ATPase domain is not necessary for male fertility
- Pms2 and Pms1 are important for Mlh1 stability in mouse thymus
- Pms2 is primarily important for Mlh1 stability in mouse testis

Figure 1.

Pms2 ATPase activity is not necessary for male fertility. **A)** Diagram of *Pms2* gene targeting. Exon 2, which contains the ATPase domain, was removed after targeting *Pms2* allele in both the *Pms2^{ko}* and *Pms2^{cre}* mice. Thus homozygous mice (*Pms2^{ko/ko}* or *Pms2^{cre/cre}*) do not have fully functional *Pms2*. *Pms2^{ko}* targeted allele has insertion of a neomycin cassette (NEO). *Pms2^{cre}* targeted allele has insertion of a splice acceptor (SA), internal ribosome entry site (IRES), Cre gene, and neomycin cassette. **B)** Images of seminiferous tubules. *Pms2^{ko/ko}* testes do not have fully functional spermatozoa, as evidenced by the lack of flagella in the lumen (arrows). *Pms2^{cre/cre}* testes look similar to *Pms2^{+/+}* testes. **C)** The total testis volume is only minimally changed in *Pms2^{cre/cre}* (n=4 testes) or *Pms2^{ko/ko}* (n=3 testes) mice compared to *Pms2^{+/+}* (n=5 testes). *Mlh1^{-/-}* testis were significantly smaller (n=3) (ANOVA p=0.05). **D)** The numbers of spermatocytes and gonias were similar between *Pms2^{cre/cre}* (n=3 seminiferous tubule sections) or *Pms2^{ko/ko}* (n=4 seminiferous tubule sections) testes compared to *Pms2^{+/+}* (n=3 seminiferous tubule

sections), but the numbers of spermatids were decreased in *Pms2^{ko/ko}* testes (ANOVA $p=0.001$).

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Figure 2.

Pms2^{cre} testes have alternative RNA splicing. **A**) *Pms2* RNA levels are no different between the different genotypes (n=2 qPCRs for each genotype). **B**) RNA levels of neighboring genes are not affected by transgene insertion (n=6 genes for each genotype). **C**) Gel showing alternative splicing in *Pms2*^{cre} testes compared to *Pms2*^{ko} testes. *Pms2*^{ko} RNA is spliced from exon 1 to 3 (loss of exon 2). *Pms2*^{cre} RNA has 2 different species, either spliced from exon 1 to 4 (loss of exon 2 and 3) or spliced from exon 1 to 5 (loss of exon 2, 3 and 4). **D**) Diagram of alternative splicing in RNA from *Pms2*^{cre} testes, which can result in an in frame RNA when spliced from exon 1 to 5 (loss of 330 basepairs). **E**) Alternative splicing from exon 1 to exon 5 in *Pms2*^{cre} results in a predicted functional protein (missing 110 amino acids in the N terminus).

Figure 3.

Images of ABI Trace files for sequencing of splice variants in *Pms2* RNA. Sequencing was performed on cDNA PCR products, which were cloned into pCR 2.1-TOPO vectors. *Pms2*⁺ allele from testes has normal splicing from exon 1 to exon 2. *Pms2*^{ko} allele from testes splices from exon 1 to exon 3 (exon 2 is deleted from the DNA). *Pms2*^{cre} allele from testes has alternative splice forms with a splice from exon 1 to exon 4 and from exon 1 to exon 5.

Figure 4.

Pms2 is important for *Mlh1* stability in mouse testis, but not thymus. **A)** Western analyses on total proteins from mouse thymi. *Mlh1* levels are not affected by a single gene knockout of *Pms2^{ko}* (n=6 thymi), *Pms1* (n=7 thymi) or *Mlh3* (n=3 thymi) compared to wild-type (WT). The double gene knockout of *Pms2^{ko}* and *Pms1* (n=6 thymi) reduced *Mlh1* stability, while *Pms2^{ko}* and *Mlh3* (n=2 thymi) had minimal effect of *Mlh1* stability. The triple gene knockout (n=5 thymi) reduced *Mlh1* levels similar to the *Pms2^{ko}* and *Pms1* double gene knockout. **B** and **C)** Western analyses on total proteins from mouse testes. **B)** *Mlh1* levels are not affected by *Pms1* (n=4 testes) gene knockout alone compared to WT. *Mlh1* levels in the testes were reduced by a single gene knockout of either *Mlh3* (n=2 testes) or, surprisingly, *Pms2^{ko}* (n=11 testes) alone. The double gene knockout of *Pms2^{ko}* and *Pms1* (n=3 testes) was not different from *Pms2^{ko}* alone on *Mlh1* stability, while *Pms2^{ko}* and *Mlh3* (n=5 testes) further reduced *Mlh1* stability compared to the single gene knockouts. The triple gene knockout (n=2 testes) reduced *Mlh1* levels similar to the *Pms2^{ko}* and *Mlh3*

double knockout. **C)** Interestingly, *Pms2*^{ko/ko} testes (n=11 testes) have a greater reduction in Mlh1 levels compared to *Pms2*^{cre/cre} testes (n=15 testes) (t-test p<0.001).

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Table 1Fecundity of *Pms2*-mutant male mice.

<i>Pms2</i> Genotype	# Males	Total # of pups	Avg. # of Pups/Litter
+/+	17	106	6.2
<i>ko</i> /+	10	61	6.1
<i>cre</i> /+	8	49	6.1
<i>cre/cre</i>	5	31	6.2