Meiotic epigenetic factor PRDM9 impacts sperm quality of hybrid mice

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Abstract

Reduced fertility of male mouse hybrids relative to their parents, or hybrid sterility, is governed by the hybrid sterility 1 (*Hst1*) locus. Rescue experiments with transgenes carrying sequences within or near *Hst1* manifested that *Hst1* contains the gene encoding meiosis-specific histone methyltransferase PRDM9. The *Prdm9* gene is responsible for partial meiotic arrest, testicular atrophy, and low sperm count in (C57BL/6J × PWD)F₁ mouse hybrids. Here we report that these male hybrids suffer an additional reproductive disadvantage, decreased sperm quality, which is (i) further exacerbated by the introduction of long transgenes carrying sequences from *Hst1* with incomplete *Prdm9* into their genome and (ii) controlled by the *Prdm9* dosage. These transgenic male hybrids displayed the features of severe oligoasthenoteratozoospermia (OAT), a human infertility syndrome characterized by a low number of spermatozoa with poor motility and morphological abnormalities. Analysis of spermiogenesis in these mice revealed acrosome detachment, aberrant elongation and condensation of the nucleus. As a result, the transgenic sperm had acrosome malformations, abnormal chromatin packaging, and fragmented DNA with elevated base oxidation, revealed by using multiple methods. Heterozygosity for one null *Prdm9* allele improved meiotic progression and sperm quality of both non- and transgenic hybrids. Our results indicate that genomic analysis of OAT patients should include consideration of allelic variants in *PRDM9*, and our transgenic models can serve as tools to understand the diverse molecular processes that, when perturbed, can cause this disease.

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Introduction

Hybrid sterility (HS) observed among male offspring of crosses between closely related animal species acts as a reproductive barrier that prevents gene flow and maintains the identity of species (Nosil & Schluter 2011). Genetic crosses using model organisms have identified several mechanisms underlying HS, including genic (e.g., speciation genes) (Mihola *et al.* 2009) and chromosomal (e.g., chromosome rearrangements) factors (Rieseberg 2001). The Dobzhansky–Muller model implies that HS is a result of incompatible interactions between divergent alleles at two or more loci (Dobzhansky 1936, Muller 1942). Thus, fertility studies in hybrids are important as they provide insights into the molecular mechanisms of speciation and reproduction.

Partially arrested spermatogenesis often results in sperm count reduction (oligospermia), sperm motility impairment (asthenospermia), and/or sperm malformation (teratozoospermia) (Yan 2009). The combination of these three phenotypes is known as oligoasthenoteratozoospermia (OAT) (Cavallini 2006). In humans, the OAT syndrome has been reported as a major cause of male infertility (Jungwirth *et al.*

2012). In the mouse, many genes are associated with OAT (Matzuk & Lamb 2008), but only three (*CMAK4*, *NANOS1*, and *TDRD6*) have been identified in humans (Khattri *et al.* 2012, Kusz-Zamelczyk *et al.* 2013, Sha *et al.* 2018). Epigenetic modifications are altered in the sperm of OAT men (Navarro-Costa *et al.* 2010, Santi *et al.* 2017), but it is unclear whether these are a direct cause or a consequence of the OAT syndrome. Thus, understanding the underlying mechanisms of OAT is of great significance for both diagnosis and treatment of this syndrome.

Strains derived from house mice (*Mus musculus*) provide useful models for understanding many areas of biology and medicine. Three closely related house mouse subspecies, *M. m. domesticus*, *M. m. musculus*, and *M. m. castaneus*, diverged from a common ancestor ~0.5 Myr ago (Geraldes *et al.* 2008). *M. m. domesticus* and *M. m. musculus* form a hybrid zone running across Europe (Sage *et al.* 1986). Males from this hybrid zone (Albrechtova *et al.* 2012, Turner *et al.* 2012) and from laboratory F₁ crosses between C57BL/6J (henceforth B6, *domesticus*) and PWD (*musculus*) display reduced fertility (Mihola *et al.* 2009, White *et al.* 2011). The causative factor of this F₁ hybrid sterility is PR-domain 9

(PRDM9), the first mammalian speciation factor identified (Mihola *et al.* 2009). PRDM9 specifies the genomic locations of recombination hotspots in mice and humans (Baudat *et al.* 2010, Parvanov *et al.* 2010, Gregorova *et al.* 2018). During meiosis, PRDM9 binds DNA via its zinc finger (ZF) domain and adds trimethylation marks including H3K4me3 and H3K36me3 to the nearest nucleosome via its PR methyltransferase domain (Baudat *et al.* 2010, Diagouraga *et al.* 2018). These actions serve to recruit DNA double-strand break (DSB) machinery to mediate DSB formation (Baudat *et al.* 2010) that initiates meiotic recombination. Comparisons between and within species (Mihola *et al.* 2009, Oliver *et al.* 2009) show that the ZF domain of PRDM9 is highly polymorphic. The quick evolutionary turnover of the PRDM9 ZFs and their binding sites leads to asymmetric PRDM9 binding and DSBs on diverged homologous chromosomes, which can be difficult to repair and thus cause fertility problems in the inter(sub)specific hybrids (Davies *et al.* 2016).

To ascertain the genetic basis of mouse HS, several bacterial artificial chromosome (BAC) transgenic mouse lines have been developed covering the male-specific hybrid sterility 1 (*Hst1*) locus on chromosome 17 encompassing several genes (Mihola *et al.* 2009). Two BACs expressing *Prdm9* rescue the meiotic progression and fertility of azoospermic (PWD × B6)_{F1} and oligospermic (B6 × PWD)_{F1} male hybrids (Mihola *et al.* 2009, Flachs *et al.* 2012). In contrast, the BAC21 transgene carrying *Fam120b*, *Psm1*, *Tpb*, *Pdcd2*, and only truncated *Prdm9* does not rescue (PWD × B6)_{F1} hybrid sterility, and further decreases the subfertility of (B6 × PWD)_{F1} hybrids due to male-specific defects in both meiosis and spermiogenesis. The introduction of BAC21 in the hybrids causes a decrease in meiotic crossover rate (but not impaired meiotic DNA repair), an increase in the number of metaphase I spermatocytes displaying univalent chromosomes, and a more frequent apoptotic cell death of the secondary spermatocytes and spermatids (Mihola *et al.* 2020). Because the lowered sperm quantity caused by partial arrest of the testis development is sometimes accompanied by decreased sperm quality, which is reminiscent of OAT, we aimed to investigate sperm of (B6 × PWD)_{F1} males with and without the BAC21 transgene and found the phenotypes observed in OAT. As reducing the *Prdm9* dosage to one gene copy rescues the sperm quantity of (B6 × PWD)_{F1} hybrids (Flachs *et al.* 2012), we analyzed the sperm quality of non- and transgenic hybrids with one *Prdm9* copy and uncovered improved sperm phenotypes. We conclude that the meiotic protein PRDM9 is important for normal sperm structure and motility.

Materials and methods

Ethics statement

The laboratory animal care and experiments observed The Czech Republic Act for Experimental Work with Animals (Decree No.

207/2004 Sb) as well as the Acts Nos. 246/92 Sb and 77/2004 Sb based on The European Union Directive 2010/63/EU. These experiments complied with the ARRIVE guidelines (Flachs *et al.* 2014) and were approved by permits Nos. 9/2016 and 16/2019 issued by the Ethics Committee for the Work with Animals of the Institute of Molecular Genetics in Prague.

Mice

The C57BL/6J strain (B6) was from The Jackson Laboratory (stock 000664). The PWD/Ph strain (PWD) was derived from wild *Mus musculus musculus* in Prague (Gregorova & Forejt 2000). Transgenic mice prepared using random integration of bacterial artificial chromosome (BAC) clones CHORI-34-289M8 (BAC21) and RP24-346I22 (BAC346) were previously described (Howell *et al.* 2005, Mihola *et al.* 2009, Mihola & Trachtulec 2017). These transgenic strains were maintained on the B6 background. Both transgenes are over 100 kb in length and carry the mouse *Psm1*, *Tpb*, and *Pdcd2* genes; BAC21 carries only the last (ZF-coding) exon of *Prdm9* and the *Prdm9* transcript encompassed by BAC346 lacks the PR/SET- and ZF-encoding domains. The BAC21 insertion site resides on chromosome 13 and segregates independently of the BAC346 insertion site (Mihola *et al.* 2020). The copy number of BAC21 is two (Mihola *et al.* 2009) and it is similar in BAC346 transgenic mice based on the testicular expression data (Mihola *et al.* 2020). The mouse *Prdm9*^{tm1Ymat} allele on PWD background has also been published (Mihola *et al.* 2019).

Sperm, spermatid, and spermatocyte phenotyping

See Supplementary methods (see section on [supplementary materials](#) given at the end of this article); the statistical significance of the data was evaluated by the generalized linear model. Each point in all figures represents an individual mouse.

Results

Poor motility (asthenospermia) of BAC21 transgenic hybrid (Tg+) mice

Among multicopy BAC transgenes developed to dissect the genetic basis of HS, only BACs carrying sequences with complete *Prdm9* rescue mouse HS (Mihola *et al.* 2009). In contrast, BACs with the same multiple gene content but truncated *Prdm9* induce partial arrest of spermatogenesis and thus lower sperm quantity of subfertile mouse hybrids (Mihola *et al.* 2020). As these phenotypes are often present along with reduced sperm quality, we investigated sperm of the transgenic and control hybrids. We obtained transgenic and nontransgenic (B6 × PWD)_{F1} mouse hybrids by crossing B6 females heterozygous for BAC21 with PWD males. As expected (Mihola *et al.* 2020), (B6 × PWD)_{F1} males heterozygous for BAC21 (henceforth denoted Tg+) had significantly lower epididymal sperm counts compared to nontransgenic (B6 × PWD)_{F1} (Tg−) and parental controls (B6 and PWD, Tgp) (Fig. 1A); Tg− males carried

less sperm than Tgp (Mihola *et al.* 2009). The semen of Tg+ mice also contained some round cells. Because the Tg+ mice were still able to produce some spermatozoa, we studied their motility using computer-assisted sperm analysis (CASA). The Tg+ sperm had ~80% reduction in both total and progressive motility when compared with the Tg- sperm ($P_1 < 0.001$, $P_2 = 0.039$, Table 1). Thus, in addition to the decreased count, the motility of sperm was also severely compromised by the presence of the BAC21 transgene.

Sperm malformations (teratozoospermia) in Tg+ mice

Normal sperm morphology is essential for fertility. To determine whether the asthenospermic Tg+ sperm

also display morphological defects, we inspected the sperm using light, fluorescence, and electron microscopy. When observed by light microscopy, most Tg- sperm displayed a normal, hook-shaped head and a long tail with a clearly visible midpiece, principal piece and end piece (Fig. 1B), but the majority of Tg+ sperm had severe head and tail defects (Fig. 1B). The Tg+ mice exhibited a 70% increase in sperm head malformations compared to the Tg- mice ($P < 0.001$, Fig. 1B and C). Three types of head deformities were distinguishable in Tg+ sperm: bent head and/or a tail coiled around the head (type 1), amorphous head (type 2), and head with reduced hooks (type 3) (Fig. 1B). Tails defects of Tg+ spermatozoa manifested as folded tails (arrowhead, Fig. 1B) or bent tails (arrow, Fig. 1B,

Figure 1 Tg+ mice are oligospermic and teratospermic. (A) Boxplots of total epididymal sperm count. Each dot represents an individual mouse in all figures. (B) Representative images of Coomassie-stained sperm from Tg- and Tg+ mice. Most Tg- sperm displayed a normal head and tail, whereas Tg+ sperm had a bent head with a tail coiled around the head (type 1), amorphous head (type 2), and reduced hook (type 3); tail abnormalities are indicated by an arrowhead and an arrow. Scale bar: 10 μ m. (C) Boxplots of the percentage of sperm showing head and tail malformations in Tgp, Tg-, and Tg+ mice. (D) Triple staining of spermatozoa with PSA (green), DAPI (blue), and MitoTracker (red) to detect acrosome, nucleus, and midpiece mitochondria in the sperm, respectively. Scale bar: 10 μ m. (E) Boxplots of the percentage of sperm showing acrosome and midpiece malformations. Two B6 and one PWD males were included as Tgp controls in (A), (C), (E), and the experiments described below using three Tgp mice. (F) Scanning electron micrographs of normal (Tg-) and abnormal Tg+ sperm heads. Scale bar: 5 μ m. Micrographs of sperm tails are shown in Supplementary Fig. 1.

Table 1 CASA results manifest asthenospermia in transgenic hybrid mice.

Genotype	n	Motility (%)	Progressive motility (%)
Tg+	3	5.7±0.6	1.3±0.6
Tg−	3	37.3±4.0	7.7±0.6
Tgp	4	67.3±2.9	25.5±4.7

Values are expressed as mean ± s.d.; n, number of males. Tgp controls included two PWD and two B6 males.

$p=0.007$). A lower percentage of Tg− spermatozoa displayed head and tail malformations, and these defects were significantly underrepresented in the Tgp sperm (Fig. 1C). We performed triple-color staining of sperm structures using pea agglutinin (PSA), which recognizes the sperm acrosome, DAPI for nucleus labeling, and MitoTracker for active mitochondria in the midpiece (Fig. 1D). The acrosomes of the Tg− spermatozoa acquired the typical crescent moon-shape, whereas the Tg+ spermatozoa exhibited various defects in the acrosome shape, size, and position (arrowheads, type 1 to 3, Fig. 1D). The acrosome malformations were increased by ~68% in Tg+ compared to the Tg− spermatozoa ($P=0.001$, Fig. 1E). The mitochondria located in the midpiece region, responsible for sperm motility, also showed various defects in the Tg+ sperm (Fig. 1D). Mitochondria were wrapped around the nucleus and contained a bulge (arrow, type 1, Fig. 1D), in some cases aggregated near the nucleus (arrow, type 2, Fig. 1D), or were weakly stained with MitoTracker (arrow, type 3, Fig. 1D). These midpiece defects were elevated by ~74% compared with those of the Tg− mice ($P<0.001$, Fig. 1E). Examination by scanning electron microscopy (SEM) (Fig. 1F) supported these findings and revealed that in one of the common deformities of Tg+ sperm, where the head was bent toward the tip of the tail (type 1), the head and the tail seemed to be connected by tissues resembling residual cytoplasm, which is normally removed during spermiogenesis (Fig. 1F).

Ultrastructure abnormalities in Tg+ sperm and spermatids

To investigate whether the poor motility of Tg+ sperm is caused by defects in the flagellum organization, we examined sperm by transmission electron microscopy (TEM) (Fig. 2 and Supplementary Fig. 2). Sections across the midpieces of Tg+ flagella showed disorganized mitochondria and irregular arrangements both of the nine outer dense fibers (ODFs) and the microtubular axoneme in the center (Fig. 2E). Both ODFs and the microtubules of the axoneme appeared to be irregular in number in the principal piece (Fig. 2F). In contrast, these flagellar components were intact in most Tg− sperm (Fig. 2B and C). The abnormal organization of these sperm tail components in Tg+ compared to Tg− ($P_1=0.039$, $P_2=0.051$) suggests a failure of Tg+ sperm tail development during spermiogenesis and likely accounts for the observed asthenospermia. Additionally, TEM confirmed that the nucleus and acrosome of Tg+ sperm were abnormally shaped (Fig. 2D) and acrosomes were detached. The nuclei showed regions of reduced electron density (arrow, Fig. 2D) in Tg+ compared to Tg− (Fig. 2D versus 2A), suggesting defective chromatin condensation. To test whether sperm defects present in the Tg+ epididymal sperm originated in the spermatids, we compared all four phases of spermiogenesis between the genotypes using peanut agglutinin (PNA) staining of testicular sections detected by fluorescence microscopy (Fig. 3A). We observed single acrosomes in round spermatids (Fig. 3A), which then grew into cap-like structures (Fig. 3A) without obvious differences in the shape between Tg− and Tg+ mice (Fig. 3B). By the third (acrosome) phase, the acrosomal structure of Tg+ spermatids failed to acquire the conical shape seen in Tg− spermatids ($P=0.044$) (Fig. 3A and C). Consequently, the acrosomes of Tg+ elongated spermatids were deformed at the last (maturation) phase compared to Tg− ($P=0.005$), which displayed

Figure 2 Sperm ultrastructure changed in Tg+ mice. (A) Normal nuclear and acrosomal morphology prevailing in Tg− sperm. Scale bar: 2 μm. (B) Mitochondria encircled the nine ODFs and axonemal microtubules in the midpiece. Scale bar: 0.5 μm. (C) FS, ODFs and Mt appeared to be normally arranged in the principal piece. Scale bar: 1 μm. (D) Abnormal morphology of the nucleus and acrosome. Scale bar: 1 μm. (E) Midpiece and (F) principal piece also displayed abnormal ultrastructure; scale bars: 0.5 and 0.2 μm. Nu, Nucleus; Ac, Acrosome; M, Mitochondria; Mt, Microtubules; FS, Fibrous Sheath.

Figure 3 Abnormal morphology of Tg+ spermatids. (A) Testis sections of spermatids stained with acrosomal marker PNA (green) and DAPI (blue), and analyzed by fluorescence microscopy. Scale bars: 10 μ m. (B) Boxplots of the percentage of spermatids with abnormal acrosomes at Golgi and cap phases, and (C) at acrosome and maturation phases. PWD males served as Tgp control. (D) TEM micrographs of Tg- and Tg+ spermatids. Tg+ mice displayed defects of spermatid structures at the late (acrosome and maturation) stages of spermiogenesis. Approximately 41% of elongated spermatids showed abnormal chromatin condensation in Tg+ males compared to 21% in Tg- (~50 elongated spermatids counted per mice, one mouse per genotype). Scale bars: 2 μ m. Arrows and arrowheads indicate the acroplaxome and the manchette, respectively. Ac, Acrosome; Nu, Nucleus.

the typical elongated, moon-shape structure (Fig. 3A and C). Differences in the acrosome shape between Tg- and Tgp spermatids were also noted at the acrosome ($P=0.025$) and maturation ($P<0.001$) phase (Fig. 3C). These results were confirmed by TEM analysis (Fig. 3D); Tg+ mice displayed detached acrosomes from nuclei, abnormal spermatid elongation and nuclear condensation during the last two phases of spermiogenesis (Fig. 3D). The acroplaxome and the manchette, cytoskeletal structures essential for acrosome attachment and sperm head shaping (Kierszenbaum & Tres 2004), were scattered in Tg+ spermatids (Fig. 3D). These data indicate that the presence of the BAC21 transgene impaired late stages of spermiogenesis resulting in morphological and ultrastructural sperm defects in the mouse hybrids.

Abnormal chromatin packaging observed in Tg+ mice

To verify the TEM data that suggested compromised chromatin condensation in the Tg+ sperm, we used Chromomycin A3 (CMA3) staining (Bianchi *et al.* 1996). We found that Tg+ mice had a 45% higher occurrence of sperm heads with CMA3-positive staining compared to the Tg- mice ($P=0.005$, Fig. 4A and B). To confirm this result, we used another parallel approach to analyze sperm chromatin condensation, flow cytometry after staining with propidium iodide (PI-FACS), a DNA intercalating agent (Vicari *et al.* 2002). The entry of PI into the nuclear DNA of some Tg+ sperm was higher compared to that of the Tg- and parental mouse sperm ($P=0.009$, Fig. 4C). Consistent with TEM results, both the CMA3 and PI-FACS analyses displayed defective chromatin condensation in sperm produced by Tg+ mice.

Figure 4 Altered chromatin condensation in Tg+ mouse sperm. (A) Chromomycin A3 (CMA3) staining in Tg- and Tg+ mice. Sperm with yellow/green fluorescence are positive for CMA3 staining (CMA3+) and indicate reduced chromatin condensation, whereas sperm with weak to no fluorescence (CMA3-) display normal chromatin condensation. Scale bar: 10 μ m. (B) Boxplots of the percentage of CMA3+ sperm in Tg-, Tg-, and Tg+ mice (means of 300 cells per animal, in triplicates). (C) Flow cytometry analysis of sperm stained with propidium iodide (PI). Some sperm from Tg+ mice were stained more strongly with PI (indicated by a higher fluorescence peak in the histogram, dashed line) than Tg- and Tg+ sperm (dotted and solid lines).

Increased DNA damage and DNA oxidation in Tg+ sperm

The abnormal chromatin packaging observed in Tg+ mice could influence the genome integrity. To test this hypothesis, we investigated the sperm DNA damage and oxidation using three approaches. To evaluate the integrity of the sperm DNA, we first utilized the alkaline comet assay (single-cell gel electrophoresis of denatured DNA). The denatured, fragmented DNA migrates farther under the influence of an electric field (generating a comet tail) than intact DNA, which remains within the confines of the nucleus (Singh *et al.* 1988, Kusari *et al.* 2017). The average level of sperm DNA damage expressed as the percentage of DNA in the comet tail was higher for the Tg+ versus Tg- mice ($P < 0.001$, Fig. 5A and B) and for parental controls (Fig. 5A). Second, we estimated the amount of oxidized bases in the sperm DNA using enzyme formamidopyrimidine-DNA glycosylase (Fpg). This enzyme recognizes oxidized pyrimidines and purines, removes these oxidized bases, and nicks DNA in the sites of the lesions. The resulting DNA nicks increase the comet tail measurable by the alkaline comet assay. The addition of the Fpg enzyme caused a ~38% increase in the percentage of DNA in the comet tail of Tg+ sperm compared to an ~7% increase in Tg- sperm ($P < 0.001$). Third, we detected a common oxidative DNA lesion,

Figure 5 Increased DNA damage and oxidation in Tg+ sperm. (A) Boxplots of the percentage of DNA in the comet tail (medians from 50 cells per male) in the control and Fpg-treated sperm. (B) Representative images of the alkaline comet assay in Tg- and Tg+ sperm. This assay revealed an increased number of DNA breaks in Tg+ sperm (note a larger comet tail) compared to Tg- sperm. Treatment with formamidopyrimidine-DNA glycosylase (Fpg) led to an increased DNA content in the comet tail compared to the control (Fpg-untreated) sperm in Tg+. Scale bar: 20 μ m. (C) Boxplots of the percentage of 8-oxo-dG positive sperm (of 200 sperm per male) in Tg-, Tg-, and Tg+ mice plus controls (no antibody and treatment with H_2O_2). (D) Immunostaining of sperm with antibody against 8-oxo-dG. Scale bar: 10 μ m.

8-hydroxy-2'-deoxyguanosine (henceforth 8-oxo-dG), in the sperm nuclei using immunostaining and found that the level of sperm positive for 8-oxo-dG was ~43% higher ($P = 0.004$) compared to the Tg- mice. The parental control sperm was similar to other WT sperm (Chabory *et al.* 2009) in displaying ~31% cells positive for 8-oxo-dG, which was significantly less compared to the Tg- sperm ($P = 0.043$, Fig. 5C). Together, these three observations indicated that the transgene presence compromised the sperm DNA integrity and increased the amount of oxidized DNA base lesions.

Reduced sperm quality of nontransgenic (B6 x PWD) F_1 males

The nontransgenic (B6 x PWD) F_1 intersubspecific hybrids (Tg-) differ from the parental strains by a lower sperm count, testicular weight (Mihola *et al.* 2009), and reduced chromosomal synapsis during pachynema (Flachs *et al.* 2012). We investigated other sperm phenotypes of Tg- males and compared them to those of their parental controls. The Tg- sperm displayed decreased motility (Table 1) and increased

malformations of the sperm head and tail ($P_1=0.013$, $P_2=0.026$, Fig. 1C). Malformations of the acrosome in elongating spermatids (Fig. 3C) and subsequent defects of both acrosome and midpiece in mature sperm ($P_1=0.043$, $P_2=0.019$, Fig. 1E), indicated that the sperm defects of the Tg⁻ mice originated from impaired spermiogenesis. While the TEM (Supplementary Fig. 2) and CMA3 analyses suggested a trend of reduced chromatin condensation compared to parental controls, the results did not reach statistical significance ($P=0.062$, Fig. 4B). Although the alkaline comet assay with/out Fpg treatment did not reveal elevated sperm DNA oxidation ($P=0.312$, Fig. 5A), the 8-oxo-dG-immunostaining (Fig. 5C) showed increased oxidation in Tg⁻ compared to Tg⁺ sperm ($P=0.043$). Thus, the nontransgenic hybrids manifested a decreased sperm quality in comparison to parental mice.

Another transgenic hybrid, BAC346, phenocopies the sperm of the BAC21 hybrid

To confirm the transgene-induced OAT causality in Tg⁺ mouse hybrids and to translate such a phenotype into mechanistic insights, we investigated whether these sperm phenotypes emerge by insertional mutagenesis. To assess this hypothesis, we used mice carrying another long transgene prepared by random genomic integration (BAC346, see Methods). This transgene carries a gene content highly similar to BAC21 (Mihola & Trachtulec 2017), including a truncated *Prdm9* lacking the PR/SET domain essential for its function. The epididymal sperm count of the BAC346 transgenic hybrid was significantly lower than that of Tg⁻ ($P=0.002$, Fig. 6A), and there were more spermatozoa with acrosome and midpiece abnormalities compared to the BAC346 nontransgenic controls ($P_1<0.001$, $P_2=0.006$, Fig. 6B). The alkaline comet assay with and without Fpg revealed elevated DNA damage ($P=0.006$) and DNA oxidation ($P<0.001$) in spermatozoa of these mice relative to nontransgenics (Fig. 6C). These analyses indicate similar sperm phenotypes of BAC346 and BAC21 transgenic hybrids, suggesting an effect independent of the insertion sites.

Reducing *Prdm9* gene dosage partially rescues normal sperm quality of Tg⁺ mice

The *Prdm9* gene contributes to genetic incompatibilities in mouse hybrids resulting in fertility reduction (Mihola et al. 2009). Manipulation of the *Prdm9* gene copy number rescues the testicular weight and sperm quantity of Tg⁻ hybrids (Flachs et al. 2012); we thus asked whether the *Prdm9* gene also acts on the sperm quality of Tg⁺ and Tg⁻ hybrids by investigating the effect of decreasing the *Prdm9* gene dosage to one copy. We generated Tg⁺ mouse hybrids with two and one *Prdm9* copies by crossing heterozygous transgenic B6 females to PWD males heterozygous for the *Prdm9* knockout (KO) allele *Prdm9^{tm1Ymat}* (PWD-*Prdm9^{KO}/PWD*; Fig. 7A). Based on this mating strategy, the F₁ generation consisted of four types of (B6 × PWD)F₁ mouse hybrids (Fig. 7A), transgenic and nontransgenic with one or two gene copies of *Prdm9*: Tg^{+/+} *Prdm9^{B6/KO}* (hereafter referred to as Tg+*Prdm9^{KO}*), Tg^{-/-} *Prdm9^{B6/KO}* (Tg-*Prdm9^{KO}*), Tg^{+/+} *Prdm9^{B6/PWD}* (Tg+) and Tg^{-/-} *Prdm9^{B6/PWD}* (Tg-). Sperm counts of Tg+*Prdm9^{KO}* males were higher in comparison with Tg+ and Tg- males ($P_1<0.001$, $P_2=0.03$, respectively, Fig. 7B). The Tg+*Prdm9^{KO}* mice had a significantly elevated percentage of motile and progressive sperm compared with Tg+ ($P_1<0.001$, $P_2<0.001$) and Tg- mice ($P_1=0.002$, $P_2=0.013$, Fig. 7C). The morphological analyses of sperm revealed restoration of the head and tail sperm defects in the Tg+*Prdm9^{KO}* mice compared to Tg+ ($P_1<0.001$, $P_2<0.001$, Fig. 7D) and Tg- males ($P_1<0.001$, $P_2=0.044$). As expected from these parameters, the transgenic hybrid males with only one copy of *Prdm9* produced offspring (7.1 ± 1.1 per female per month). The decrease in the *Prdm9* gene dosage normalized the sperm count of Tg+, so that Tg+*Prdm9^{KO}* was comparable to Tg-*Prdm9^{KO}* ($P=0.762$, Fig. 7B) and to the PWD parental control ($P=0.987$). However, sperm motility and morphology were different between Tg+*Prdm9^{KO}* versus Tg-*Prdm9^{KO}* ($P_1=0.054$, $P_2=0.006$) and versus Tg⁻ ($P_1=0.014$, $P_2=0.023$; Fig. 7C and D), suggesting that these sperm phenotypes were affected by the transgene presence. No significant differences were observed in the sperm count, motility and morphology between Tg-*Prdm9^{KO}* and parental controls (Fig. 7B, C and D).

Figure 6 The effect of transgenes on hybrid sperm is not limited to BAC21. BAC346 transgenic (B6 × PWD)F₁ hybrids (Tg⁺) display (A) Decreased sperm count, (B) increased percentage of sperm with acrosome and midpiece malformations compared to the Tg⁻ controls. (C) The alkaline comet assay uncovered more DNA breaks (light grey) and the comet assay with Fpg showed more DNA oxidation (black) in BAC346 Tg⁺ versus Tg⁻ sperm.

Figure 7 Sperm quantity and quality is affected by epigenetic factor PRDM9. (A) Mating strategy to generate (B6 × PWD)_{F1} non/transgenic mouse hybrids with varying gene dosage of *Prdm9*. (B) Boxplots of sperm counts in mouse hybrids and TgP controls. (C) Boxplots of the percentage of motile and progressive sperm. (D) Boxplots of the percentage of head and tail malformations.

Since the decreased *Prdm9* dosage in (B6 × PWD)_{F1} hybrids improves meiotic progression by promoting efficient synapsis and sex body (SB) formation (Flachs *et al.* 2012), we tested whether this mechanism can also explain the higher sperm count and quality in Tg+*Prdm9*^{KO}. We analyzed nuclear spreads of Tg+*Prdm9*^{KO} and Tg+ testes immunostained for markers of chromosomal synapsis (SYCP3, SYCP1) and SB formation (γH2AX) (Fig. 8A). Homologous autosomes were fully synapsed and SB formed (encompassing just sex chromosomes) in ~58% of pachytene nuclei from Tg+*Prdm9*^{KO} compared to only ~45% in Tg+ males (*P*=0.032).

Meiosis progression requires proper establishment of the pericentric heterochromatin (PCH) structure (Takada *et al.* 2011), as its defects lead to asynapsis of mouse chromosomes (Takada *et al.* 2011), and the loss of PCH histone modification marks (e.g., H3K9me3) disrupts heterochromatin and genome stability (Peng & Karpen 2009). These meiotic defects are similar to those seen in Tg+, we hypothesized that the Tg+ mice might have defective PCH and tested whether PRDM9 affects this structure. We observed a similar H3K9me3 signal distribution in Tg+*Prdm9*^{KO} and Tg+ mice detectable at the DAPI-rich PCH regions in meiotic and post-meiotic cells, indicating that PRDM9 did not affect this PCH histone modification pattern (Fig. 8B). These results may suggest that the chromatin defects observed in elongating spermatids and mature sperm of Tg+ mice were not the result of large-scale defects in the PCH structure. In conclusion, the reduced *Prdm9* dosage improved sperm motility and morphology in both non- and transgenic hybrids, likely due to more successful execution of meiotic events such as synapsis and SB formation.

Discussion

Histone-3-methyltransferase PRDM9 has a well-documented role in _{F1} hybrid sterility (Mihola *et al.* 2009) and in the initiation of meiotic recombination in mammals (Baudat *et al.* 2010). We show here, using a reduced *Prdm9* dosage, that PRDM9 levels are important not only for meiotic progression and sperm quantity, but also for the sperm motility and morphology of (B6 × PWD)_{F1} hybrids. This decreased level of PRDM9 protein probably counteracts the negative effects of *Prdm9* interaction with loci that impair spermatogenesis progression of mouse hybrids, should it be the differentially eroded meiotic recombination hotspots (Davies *et al.* 2016) or other loci. The genomic region of 62 megabases carrying *Prdm9* affects the number of round spermatids and the presence of multinuclear cells in mouse _{F2} hybrids (Schwahn *et al.* 2018). Spermiogenesis defects (Fig. 3) were found in both Tg- and Tg+ mice, but their effects on spermatozoa were completely or partially rescued by a decreased *Prdm9* dosage (Fig. 7), suggesting an additional postmeiotic role of PRDM9. Because PRDM9 expression is restricted to spermatocytes during meiotic prophase I (Sun *et al.* 2015), it seems unlikely that it could directly influence spermatid differentiation. For instance, chromatin compaction appears to be affected in elongating spermatids undergoing nuclear condensation, a process that requires proper postmeiotic gene expression to ensure histone replacement by protamines (Dowdle *et al.* 2013). We speculate that the molecular interactions of PRDM9 with other chromatin remodelers (Parvanov *et al.* 2017) linked to

Figure 8 Chromosomal synapsis but not heterochromatin formation of Tg+ spermatogenic cells depends on *Prdm9*. (A) Representative images of nuclei from adult testicular spreads indirectly labeled using antibodies against synaptonemal complex proteins (SYCP1 and SYCP3), centromeres (CENT), and DSBs under repair (γ H2AX), and counterstained for DNA with DAPI. The upper images show a Tg+ pachytene nucleus (50-70 counted per each of 5 animals) with two unsynapsed autosomes encompassed in the SB. The lower images capture a completely synapsed (normal) pachytene nucleus from Tg+*Prdm9*^{KO}. In total, 50-70 nuclei from Tg+*Prdm9*^{KO} were analyzed per animal (4 animals). Scale bars: 10 μ m. (B) Adult nuclear spreads stained with DAPI and immunolabeled for heterochromatin marker H3K9me3, synaptonemal complex, and centromeres. In all 20 spermatocytes and 211 round spermatids investigated from both Tg+ and Tg+*Prdm9*^{KO}, the DAPI-rich signal co-localized with the H3K9me3 signal as in these four representative cells. Scale bars: 10 μ m.

histone replacement (e.g., CDYL) (Liu *et al.* 2017) in the repaired recombination sites may influence subsequent chromatin compaction events. Alternatively, chromatin defects observed in elongated spermatids may be due to compromised spermiogenesis rather than a specific PRDM9-related effect.

Our Tg+ mouse hybrid model recapitulates all three characteristics of the human OAT: (1) low sperm output; (2) poor motility; and (3) head and tail structural abnormalities. The OAT phenotype has been previously reported in at least eleven types of mutant mice (Supplementary Table 1). Four of these reports did

not investigate the sperm chromatin condensation, three found it intact, and four detected impaired chromatin condensation (Supplementary Table 1). Only one of these eleven reports described the analysis of the DNA integrity of spermatozoa and found it intact (Liu *et al.* 2018). Such phenotypic variability among the mouse models and human patients suggests that different mechanisms may underlie the OAT subtypes. Thus, Tg+ represents an animal model to study certain types of unexplained OAT cases in men, displaying sperm chromatin defects and DNA damage (Liu *et al.* 2004, Rahiminia *et al.* 2018).

Defective chromatin condensation within the sperm nucleus, such as that observed in both mice and humans with protamine deficiency, is associated with DNA damage and fertility problems (Cho *et al.* 2003, Torregrosa *et al.* 2006). Our findings support the idea that improper chromatin condensation in sperm manifests with DNA damage and oxidation, thereby impairing sperm quality (Figs 4 and 5). We observed sperm with deformed nuclei and acrosomes, the acrosomes being frequently detached from the nuclei. The malformations of both the nucleus and the acrosome are common phenotypes in some OAT men (Reichart *et al.* 2000, El-Kamshoushi *et al.* 2013). These abnormalities of the sperm head structures appear to be common for a variety of genetic defects that perturb the structure and function of the mouse acrosome-acroplaxome-manchette complex (Kierszenbaum & Tres 2004, Akhmanova *et al.* 2005, Zhou *et al.* 2009). In line with these studies, we noticed that this complex was impaired in Tg+ mice during spermiogenesis, and as a result might be involved in the cascades that lead to sperm defects. The manchette also contributes to the transport of proteins necessary for the assembly of axoneme and ODFs into the growing sperm tail via a process known as intramanchette transport (Kierszenbaum 2002, Kierszenbaum *et al.* 2011). Abnormal mitochondria arrangement and/or axoneme structure have been reported in some human OAT patients (Baccetti *et al.* 2006, Mitchell *et al.* 2006), suggesting that a similar mechanism may underlie these malformations in mice and humans.

Because the transgenic mouse hybrids share the same genetic background with the non-transgenics, the OAT phenotype severity observed in the Tg+ males is likely a consequence of the transgene. Although the overall effect of the transgene presence exacerbated the sperm quantity and quality, some spermatozoa displayed normal morphology and motility, consistent with some males producing a few offspring (Mihola *et al.* 2020). The integration sites for BAC21 and BAC346 segregate independently (Mihola *et al.* 2020), and insertional mutagenesis thus cannot explain the OAT phenotype.

Deletion of a *Prdm9* copy rescued both sperm count and sperm quality in Tg− males, but the Tg+ sperm quality was rescued only partially (Fig. 7), indicating that some sperm parameters are controlled by the transgenes via a mechanism independent of *Prdm9*. The phenotypic similarity of BAC346 and BAC21 transgenic hybrids and the finding that the sperm count correlates inversely with increased *Psmb1* mRNA levels encompassed by both BACs (Mihola *et al.* 2020) support the role of common genes expressed by both transgenes in the observed sperm phenotypes of these mice. PSMB1 and other proteasomal proteins were found to be overexpressed in the sperm of OAT men (Agarwal *et al.*

2015), suggesting that enhanced proteasome activity may cause, accompany, or exacerbate this disorder.

Sperm head and tail malformations as well as reduced motility have been detected and genomic loci mapped in mouse hybrids, see for example, (Larson *et al.* 2018), but here we describe the malformations in finer detail. The refinement was possible due to the enrichment of these sperm defects in Tg+ hybrids. Our results establish a novel role for PRDM9 in regulating normal sperm quality of hybrids and provide a foundation to further elucidate its mechanism of action. While caution is vital before extrapolating from mouse to human disorders, our study urges an investigation of whether coding or regulatory variants in the *PRDM9* gene are present in patients with idiopathic OAT.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0528>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F K and Z T designed the experiments. F K performed sperm phenotyping, testis histology, and statistics; O M executed nuclear spread experiments. J C S contributed reagents. O M and J C S contributed discussion. Z T supervised the study. F K, J C S, and Z T wrote the paper.

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