Opponent´s review

Thesis: Male infertility and DNA germ cell breaks affected by the epigenetic factor PRDM9

Type: Ph.D. Dissertation

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Ph.D. study program: Molecular and Cellular Biology, Genetics and Virology

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Opponent: doc. MUDr. František Liška, Ph.D.; Charles University, 1st Faculty of Medicine

General Remarks

Form

The reviewed thesis is a full-format dissertation written in English, on 101 pages with typical structure including Abstract (which is also translated into Czech), Introduction, Aims and Motivation, Materials and Methods, Results, Discussion, Conclusion, Author´s publications and References. The number of figures is a little hard to count since some figures come (in a little unusual way) in two or more separate parts, but following the "main" numbering, introduction contains 5 figures and Results 18. There are 158 references (counted by doi.org occurrences). There are also two tables in Results, and one unlabeled as such in Materials and Methods. The thesis reads well, with minimum typographical errors, and the author´s results and opinion are easily understandable. As I am not a native speaker, I cannot fully judge the formal grammar and style regarding proper English usage, but I do not identify anything "unusual".

Content

The thesis investigates the mouse hybrid sterility locus 1 (*Hst1*) beyond the known meiotic function of *Prdm9*. First, using BAC transgenes alone or in combination with *Prdm9* heterozygous loss of function, the author demonstrates a detrimental postmeiotic effect of the copy number gain of genes in the *Hst1* locus except of *Prdm9*, including abnormal nucleus condensation (with DNA fragmentation and oxidative damage), abnormal axonemal structures and acrosome – leading to a oligoasthenoteratozoospermia-like syndrome. Although there is an evident candidate gene that can cause the abnormality, *Psmb1*, coding a proteasome subunit, intriguingly, decrease of *Prdm9* dosage (heterozygous knock-out) alleviates many of these abnormalities, which suggests that the situation is more complicated.

Indeed, second, the author characterizes in detail the reproductive phenotype of *Prdm9* knockout PWD mouse and SHR rat, which show, in addition to a partial meiosis arrest (attributable to apoptosis of spermatocytes in pachytene and metaphase I stages, and in agreement of the known role of PRDM9 in meiotic crossing-over initiation), also a rare type of teratozoospermia, the acephalic spermatozoa syndrome. The author shows that a detachment of the basal plate from the remainder of the nucleus (basal plate is assembled on the nuclear membrane(s)) can be the reason of the neck fragility. This could indicate a failure of the LINC (linker of nucleoskeleton and cytoskeleton) complex (itself a known cause of acephalic sperm – see *Sun5* loss of function). That could prove important for deciphering the molecular mechanism, since it is really hard to imagine, how a histone modifying enzyme PRDM9 expressed only during the early prophase of meiosis I can impact postmeiotic events.

However, impaired chromatin remodeling can in principle, while occurring in meiosis, later compromise attachment of the nuclear cytoskeleton, which could interrupt the chain of connections holding the nucleus and flagellum together under mechanical stress. From the above description, it is clear that I like the work very much and regard its original findings as scientifically important. I have here to admit a potential conflict of interest, as I was involved in analysis of a rat mutant with loss of centrobin function, which also leads to acephalic spermatozoa syndrome (centrobin is a structural component of the head to tail coupling apparatus). My training was genetics, not histology, therefore I cannot fully appreciate the amount of work necessary to accomplish the results; however, especially the analysis of all cell types in all stages of the rat spermatogenesis is in my opinion a histological "tour de force".

Specific questions or remarks

Methods

1) It is typical for reviewers of diploma and dissertation theses to criticize the statistical methodology. I will not be an exception. Maybe the author planned it thus, since Section 3:19, Statistical analysis, is clearly a stump. It could be suitable for a journal publication (not if I was the reviewer though), but in a dissertation thesis it deserved obviously more attention. For example: Which independent variables/factors were used to build the general linear models? Which prerequisites were tested using the residual analysis, and how data were normalized when the prerequisites were not met? Logarithm transformation is mentioned as a normalization method, but was it always successful, so that nonparametric statistical methods could be avoided? What correction method for multiple comparisons was employed? .

Results

2) Fig. 4.4C – FACS with cells stained using propidium iodide

   I cannot identify which line is corresponding to which genotype. The legend says:

   "The higher fluorescence peak in the histogram (dashed line) reflects increased PI staining for Tg+ sperm compared to Tg− and Tgp spermatozoa (dotted and solid lines)."

   But I see the dashed line to peak at cca 100 nominal fluorescence intensity, the peak (number of cells) being about 1/3 of the highest (solid line), and comparable to the grey line peak. The grey line (maybe it was the originally dotted one) has another peak with higher fluorescence intensity (approx. 1000). which is almost or completely missing in the other samples. This is probably a different cell type, e.g. somatic (diploid) cells? I think that the legend should be here more clear and comprehensive.

Discussion (or general questions)

3) *Psmb1* was identified as a candidate gene for the *Prdm9* independent effect on spermatogenesis observed in the BAC transgenes. Since the effect seems to be dependent on the *Psmb1* transcript level, is it planned to dissect the possible role of this gene dosage by a transgenic experiment?

4) In comparison of the acephalic spermatozoa in Prdm9-/- SHR rats and PWD mice the milder phenotype in SHR was attributed to species difference – longer time and more discernible stages in the rat spermiogenesis. While this is a valid assumption, I would lile to note here, that in rats with mutant centrobin, the headless tails do contain the cytoplasmic droplets, as do the PWD

Prdm9 mutants, while in centrobin mutants where the mutation is partially rescued by transgenic centrobin, the still decapitating sperm are much less abnormal with no visible retained cytoplasmic droplet. This speaks more for a profound species dependence, just a different severity scale.

5) As author notes, *Prdm9* is expressed (latest) in the prophase of meiosis I, while this work catalogs the postmeiotic phenotypes. In Prdm9-/- rats and mice, this is even the major affliction. Of course the obvious question is: How the meiotic gene defect drives postmeiotic effects? This question was asked but could not be answered in this work. Is there any vision about the possible mechanisms or regarding the way to tackle this problem?

Minor remarks

Abstract

"However, reciprocal (B6 × PWD)F1 hybrids and some rodent models lacking PRDM9, i.e., PWD and SHR rat males execute meiotic recombination, produce sperm, raising the possibility that PRDM9's role may extend beyond meiosis."

In this important statement, information is lacking that these animals while producing sperm, still suffer from sub- or infertility (when first reading this I was wondering why to study these models when the animals are fine).

Introduction

"Mammals have evolved intricate...spermatogenesis"

While in other animals it does not exist or it is comparatively simple?

"but only three (NR5A1, DMRT1, and TEX11) have identified in human infertility cases (Tuttelmann et al. 2018)."

This statement is quite outdated. Only for the acephalic spermatozoa syndrome, a rare form of teratozoospermia, also discussed in this work, there are five genes already ascertained with 2 or more unrelated patients (*SUN5, PMFBP1, TSGA10, BRDT* and *HOOK1* [1-6] etc.)Also the previous reference to 600 mouse models could be reestimated, e.g. using databases like MGI.

"from...azoospermia to suboptimal sperm quality"

What about suboptimal sperm quantity?

Fig. 1: Source is missing (or a statement that the graph was developed by the author).

1.1

Molecular biology of meiosis entry is well described, should the stem cell maintenance and proliferation be also discussed or the reader referred to appropriate reviews?

1.2

How the centromeric cohesins are protected against separase cleavage in anaphase I?

1.3

A question can be raised whether genes/proteins involved in HTCA should not be described in more detail.

1.4

“Methylation of histone lysine (K) residues alters chromatin structure”

Please do not forget other histone modifications + DNA methylation.

1.6

“It has been shown that mouse intersubspecific F1 hybrids display a higher level of copy number variations (CNVs) compared with the parental subspecies”

This is not easily understandable claim, since parental strains are inbred so there are almost no variants, while F1 hybrids will be heterozygous for multiple variants, be it SNVs or CNVs. However, the referenced article finds de novo CNVs arising in F1 hybrids by presumably intrachromosomal "unequal crossing over" leading typically to expansion of the number of the repeat units compared to the original alleles. Therefore this should be explained in a clearer way for typical readers like me to understand.

Methods:

3.3

“ ...containing (in mM): 119. 37 NaCl.”

Could you demonstrate you can reproducibly achieve 10 µM accuracy in NaCl concentration?

3.15

“After rising slides in neutralizing buffer”

Rinsing?

3.17

"...mouse testes were de-capsulated and dissociated to single cell suspension by sequential enzymatic digestion with collagenase (Sigma, cat. No. C2674) and DNase (Roche, cat. No. 11248932001) in enriched Krebs-Ringer (EKRB) buffer containing (in mM): 120.1 NaCl, 4.8 KCl, 25.2

NaHCO3, 1.2 KH2PO4, 1.2 MgSO4, 1.3 CaCl2, 11.1 glucose, 1 glutamine, 10 ml l-1 essential amino acids and 10 ml l-1 non-essential amino acids as described (Anderson et al. 1999). Following release of germ cells in suspension, a small aliquot of 0.1 M sucrose solution containing germ cells was dropped onto glass slides ..."

I think a pelleting and resuspension step is omitted here, or how were the cells transferred from EKRB to 100 mM sucrose?

Results

Fig. 4.1B + text on p. 50

“...had reduced apical hooks (type 3) (Fig. 4.1B)."

To me, it seems that the hook looks quite fine, the abnormality being a kinked tail (arrow in Fig. 4.1B points to that). Figs4.1D+F show the hook reduction though in "type 3".

4.2.

"These peri-axonemal structures provide the energy necessary for axoneme bending and thus flagellar beating. In addition, they offer mechanical support and thereby stabilize flagellar motion (Lindemann and Lesich 2016)."

Wouldn´t it hurt to say that the mitochondria provide the energy and all periaxonemal structures provide the mechanical support? There can be of course a long discussion whether these structures provide elastic energy, but that has to be loaded into them by utilizing the mitochondrial ATP in active axomenal stroke anyway.

4.4.

CMA3 assay (also Fig. 4.4).

Where is the line between CMA3 positive and CMA3 negative sperm nuclei? It seems to me rather subjective.

Fig. 4.5. Why is the 8-oxo-dG staining concentrated in the posterior part of the nucleus?

4.6.

It should be stated more clearly that in this section previously presented data are reviewed in terms of differences between Tgp and Tg- groups. Also, "Table 1" is probably referring to Table 4.1.

Fig. 4.8. - For readers who first peruse the figures it would be helpful in Fig. 4.8A to include both *Prdm9* and transgenic genotypes in parental strains too (otherwise the "sudden occurence" of *Dom2* allele in F1 can be confusing).

Fig. 4.11, 4.11´, 4.11´´ - I have to say that I hate this numbering system.

4.12

"Retained mutant spermatid heads were discovered in stages IX (3.5 ± 0.68 versus 0.3 ± 0.1 P =0.015) and X (3.4 ± 0.4 versus 0.5 ± 0.3 P =0.005), whereas controls displayed rare S19 retention." "S19" probably means stage 16 spermatid heads, as this happens in the mouse.

Fig. 4.17B, wild-type stage XIV.

The cell labeled MII (metaphase II spermatocyte) does not look as having a metaphase plate chromosomes - could it be a secondary spermatocyte prior to metaphase?

Conclusion

The thesis is well-written and contains scientifically sound and novel original results, corroborated by two recent full-text publications in reputable scientific journals (once as first author). Therefore I fully recommend this thesis for (successful) defense and subsequent graduation of the author, with the Ph.D. title.

In Prague, 30th of August 2021

František Liška

1 Chen, H. *et al.* Detection of heterozygous mutation in hook microtubule-tethering protein 1 in three patients with decapitated and decaudated spermatozoa syndrome. *J Med Genet* **55**, 150-157, doi:10.1136/jmedgenet-2016-104404 (2018).

2 Li, L. *et al.* Whole-exome sequencing identified a homozygous BRDT mutation in a patient with acephalic spermatozoa. *Oncotarget* **8**, 19914-19922, doi:10.18632/oncotarget.15251 (2017).

3 Sha, Y. W. *et al.* TSGA10 is a novel candidate gene associated with acephalic spermatozoa. *Clin Genet* **93**, 776-783, doi:10.1111/cge.13140 (2018).

4 Xiang, M. *et al.* Pathogenesis of acephalic spermatozoa syndrome caused by splicing mutation and de novo deletion in TSGA10. *J Assist Reprod Genet*, doi:10.1007/s10815-021-02295-x (2021).

5 Zhu, F. *et al.* Mutations in PMFBP1 Cause Acephalic Spermatozoa Syndrome. *Am J Hum Genet* **103**, 188-199, doi:10.1016/j.ajhg.2018.06.010 (2018).

6 Zhu, F. *et al.* Biallelic SUN5 Mutations Cause Autosomal-Recessive Acephalic Spermatozoa Syndrome. *Am J Hum Genet* **99**, 1405, doi:10.1016/j.ajhg.2016.11.002 (2016).