

Peer Review File

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Reviewer A

In the first part of their review, authors provide a summary of the pre- and post-fertilization function of protamines, as well as their transcriptional, translational, post-translational and epigenetic regulation. Most interestingly, in the second part, actual and possible future clinical and laboratory applications are presented. This represents highly important information for the scientific community, as to date, protamines are well-known for scientists/doctors working in the reproductive field, while these molecules are nearly unknown in all other fields - despite their great potential for both laboratory and clinical application.

The review presents a highly important topic, however, needs thorough revision before publication.

General comments: Be more precise!

The manuscript has a focus on protamine-1. This is ok, as it is ubiquitous, smaller and better for handling than protamine-2. However, when talking about 'the protamines,' it has to be kept in mind that P2 is different from P1. This must be distinguished throughout the text: state clearly whether it's general to protamines, or specific for protamine-1.

In addition, the text must be more precise concerning the species that is talked about. Firstly, there are differences between species. Secondly, it's not in the responsibility of the readers to check all the original literature what species has been used.

(Minor comment: I would prefer application or usage instead of utilization, however, this is a matter of opinion).

Reply: we accepted the comments.

Changes in the text: Moreover, we will discuss the potential clinical utilization of PRM1 in human malignancies.

In human, it is reported that a programmed protamine-to-histone exchange occurs immediately after the paternal chromatin enters in the egg (35-37).

Specific comments:

Abstract

Comment 1: Instead of protamine proteins are specialized in sperm I would prefer protamine proteins are specific for sperm.

Reply 1: we accepted the comments.

Changes in the text: The protamine proteins, which are specific in sperm and play essential roles in fertilization, are characterized by an arginine-rich core and cysteine residues. (See Page 2, line 1).

Comment 2: There seems to be some confusion, as PRM1 is NOT expressed solely in specific species. This is the case for PRM2, whereas PRM1 is expressed in all vertebrates / most species analyzed so far. In non-vertebrates, sometimes these molecules are not called protamines, but reveal exactly the same function.

Reply 2: we meant that only PRM1 is expressed in the sperm of some species, PRM2 or PRM3 is absence. However, the expression was wrong.

Changes in the text: In addition, the abnormal expression or proportion of the two protamines PRM1 and PRM2 is known to be associated with subfertility and infertility, especially for PRM1 which is highly evolutionary conserved in mammals and expressed solely in specific species in all vertebrates. (See Page 2, line 4 and 7).

Introduction

Comment 3: When DNA AND histones/protamines are described, chromatin should be used instead of DNA-only or genome (throughout the text).

Reply 3: we accepted the comments.

Changes in the text: PRMs bind to DNA leading to condensation of the spermatid genome chromatin and a genetically inactive status (1). (See Page 4, line5);

a programmed reversal of protamine-to-histone exchange occurs immediately after the paternal genome chromatin enters in the egg (Page 8, line 10);

which is a prerequisite for paternal genome chromatin decondensation in the fertilized egg and subsequent zygotic development (Page 12, line 1);

When the overexpressed PRM1 protein entered the nuclei, histone-protamine exchange occurred in the chromatin which was formed into condensed station (Page 15, line 12).

Comment 4: It's right that protamines are typically short proteins, however, 50-100

amino acids is only true for protamine-1, while protamine-2 is in most cases 3-4 times larger than protamine-1.

Reply 4: It is true that protamine-2 is larger than protamine-1. Whereas protamine 2 is initially 103 amino acids and undergoes N-terminus cleavage to a mature protein of 57 amino acids in sequence. So, in the text, we meant mature protamines.

Changes in the text: Compared with histones and protamine-like proteins, mature protamines are typically short proteins (50-100 amino acids) with higher amount of arginine (up to 70%) and two structural elements identified in all vertebrates. (See Page 4, line 17).

Pre-fertilization stage

Comment 5: Two-week long spermatogenesis must be specified - which species? For example, in man, it's 64 days. It cannot be expected that readers have to look up the original articles.

Reply 5: we accepted the comments.

Changes in the text: Generally, during spermatogenesis, the mRNA of TPs and PRMs are stored in ribosomal protein granules of cytoplasm after transcription, followed by translation into proteins, and participate in sperm prolongation and spermatid differentiation (29, 30). (See Page 7, line1).

Post-fertilization stage

Comment 6: Why always 'reversal of histone-to-protamine exchange' and not directly protamine-to-histone exchange/replacement?

Reply 6: we accepted the comments.

Changes in the text: In human, it is reported that a programmed protamine-to-histone exchange occurs immediately after the paternal chromatin enters in the egg (see Page 8, line8, 9);

Noteworthily, the protamine-histone exchange has inspired more and more researches of PRM1 in other areas (see Page 8, 18 and 19).

Figure 1

Comment 7: The 1-letter code 'Q' stands for glutamine, not glytanyl! The acetyl-group would be glutaminyl. Glutamyl belongs to glutamine acid, abbreviation 'E.' Please correct. In addition, I suggest to mark N- and C-terminus of the protein.

Reply 7: we accepted the comments.

Changes in the text: Fig. 1 General view of three domains for PRM1 protein. Amino

acids represented by letters: M, Methionine; A, alanine; C, cysteine; H, histidine; M, Methionine; P, proline; Q, glutaminate; R, arginine; S, serine; T, threonine; Y, tyrosine; This diagram does not represent the spatial structure of the protein. (See Page 19, line3).

Figure 2

Comment 8: I cannot image that anyone will understand this Figure?! Why 2 times protamine on the right side? Small figure in the lower right corner, text is too small, unreadable. Upper line, expression regulation, tells the reader absolutely nothing, main text is much better, this part in the Figure is confusing. Resumé: either delete or restructure. Maybe compare application in the past with future new possibilities of application.

Reply 8: we accepted the comments.

Changes in the text: we have modified our text as advised (see Page 20).

Reviewer B

Ren et al provide a short but comprehensive review of all relevant aspects of the biological role of protamine 1 in gametogenesis, in tumorigenesis and as a mechanism for nuclear reprogramming and drug delivery. The review is well structured with meaningful paragraph headings. The review is written mostly in good scientific language, the references are correct and well chosen.

However, I have a few comments on the review:

Comment 1: This is a narrative review. Accordingly, a methodological part describing the method and results of the literature research the review is based on is not mandatory. However, the authors added two tables listing references of regulation mechanisms of expression and potential applications of PRM1 without further information. I strongly recommend to remove these tables adding the according references to the text or to add additional information (not just target gene or one keyword on application) to the tables.

Reply 1: we accepted the comments.

Changes in the text: we have modified our text as advised.

Comment 2: Figure 1 shows the amino acid structure of PRM1. There are many

options to use this figure for additional helpful information (spatial structure, AA sequence of PRM2, interaction with DNA, cystein bridges within and between PRM1 molecules). I recommend to modify this figure accordingly.

Reply 2: we accepted the comments.

Changes in the text: we have modified our text as advised by Reviewer A (see Page 19).

Comment 3: Figure 2 is supposed to give an overview more or less over the content of the whole review. This figure adds nothing to the understanding of the addressed aspects and appears rather confusing. There are multiple options to add graphical information more helpful than with this figure. It should be removed.

Reply 3: we accepted the comments.

Changes in the text: we have modified our text as advised by Reviewer A (see Page 20).

Comment 4: Corrections of language are recommended in the first sentence of the paragraph 'Clinical utilization of PRM1' and the conclusion. The authors should decide if the plural of an abbreviation is written with or without 's'; both is possible but should be used consistently throughout the text. Furthermore, formatting of gene and protein names should revise throughout the manuscript.

Reply 4: we accepted the comments.

Changes in the text: The human PRM1 and PRM2 genes co-locate in a tight cluster on chromosome 16 at 16p13.2 (see Page 5, line 8);

Since protamine proteins participate in the late stage of spermatogenesis, abnormality of PRM1 expression is responsible for spermatogenic qualitative defects. (Page 12, line 22).