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Differential expression of the nucleolar protein fibrillarin during mammalian spermatogenesis and its probable association with chromatoid body components

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ABSTRACT

Chromatoid body (CB) is a cytoplasmic structure of male germ cells that has been indicated as having a role in the RNA and protein storage for the final differentiation of spermatozoa. Recent studies have indicated that some of these macromolecular complex components have nucleolar origin. The aims of the present study were to monitor the expression of fibrillarin nucleolar protein in mammalian seminiferous tubules at different stages of the spermatogenic cycle; to check fibrillarin distribution during the CB assembly; and also its interaction with two well-known CB markers (MIWI and HSP70). Seminiferous tubules were isolated by transilluminating microscope from testis of adult mice. Fibrillarin expression and also co-localization between fibrillarin and MIWI/HSP70 were performed by Western blot (WB) and by immunofluorescence (IF), respectively. Total proteins from testis of adult mice were also used to perform co-immunoprecipitation (Co-IP) experiments. Our results demonstrated higher fibrillarin expression in seminiferous tubules in stages IV–VI, and a close localization of fibrillarin with MIWI (a protein that plays a role in RNA metabolism in the CB), as well as with HSP70 (a protein that plays a role in the proteasome folding in the CB). We also performed Co-IP between fibrillarin/MIWI and between fibrillarin/HSP70 in order to determine whether MIWI or HSP70 interacts with this nucleolar protein. We found MIWI in the Co-IP precipitate, but not HSP70. In conclusion, our results show that fibrillarin may participate in the physiological activities performed by the CB by interacting with CB components that play a role in RNA metabolism.

1. Introduction

Every animal germ cell possesses a set of cytoplasmic material known as *nuage*. The polar body of oocytes in species from the genus *Drosophila* is likely the most commonly known example of nuage. In male mammalian germ cells, the nuage is more commonly referred to as the *chromatoid body*, or CB (Parvinen 2005). The CB is a cytoplasmic structure that seems to play a role in storing mRNA and proteins that will, in turn, play a role in final spermatozoid differentiation (Söderström and Parvinen 1976; Saunders et al., 1992). The CB can be detected in the cytoplasm of primary spermatocytes in the pachytene stage. In this type of cell, the CB is a fibro-granular structure formed by mitochondria. At the end of meiosis, the mature CB condenses into a fibrous and lobed granule and moves around the surface of the round spermatid nucleus. This structure remains in the cytoplasm of the spermatid until the nucleus begins to elongate. Finally, it disappears during the end stages of spermiogenesis (Wang et al., 2015).

The CB is considered to be a macromolecular complex that seems to play the role of coordinator of the post-transcriptional expression of gene products in male haploid germ cells and of an mRNA processing site (Kotaja and Sassone-Corsi 2007). Some of the molecular components that make up the CB, such as DNAse, RNAse, AcPs, ubiquitin, and HSP70, have been found to cause this structure to act as a degradation site where DNA, RNA, and proteins that are no longer necessary for the spermatogenesis process are degraded (Haraguchi et al., 2005).

Some authors have proposed that the CB originates from nucleolar material that is fragmented in the early stages of sper-

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matogenesis and then migrates to the cytoplasm, thus forming the small nuages that slowly coalesce in order to form the structure known as the CB (Comings and Okada 1972; Andersen 1978; Takeuchi and Takeuchi 1990; Andonov 1990; Peruquetti et al., 2012; Peruquetti and Azeredo-Oliveira 2013). In a recent study in which the authors sought to associate the fragmentation of nucleolar material during meiotic cell division with the formation of the CB in the initial spermatids of triatomine insects, the presence of fibrillarin, a nucleolar protein, was detected in a perinuclear cytoplasmic position that corresponds to the location where the CB is frequently observed (Silistino-Souza et al., 2012). This possible location of fibrillarin in the molecular composition of the CB of insects provides more evidence for the hypothesis that nucleolar products may be associated with CB during spermatogenesis. In addition, another nucleolar protein — C23/nucleolin — was detected in the CB after an extensive proteomic analysis of this structure (Meikar et al., 2014). Although the study mentioned here did not detect the presence of fibrillarin in the CB's molecular composition, the authors did identify a large number of transient proteins, or proteins having temporary nuclear function/localization before being translocated to the CB. A study that analyzed the nucleolar cycle during gametogenesis demonstrated that the reestablishment of nucleolar morphology during yeast gametogenesis is an important factor in the removal of cells that are damaged as a result of aging (Ünal et al., 2011). Therefore, it can be inferred that some nucleolar proteins may be shuttled to the CB during spermatogenesis as part of the nucleolar morphology reestablishment process.

In addition to its role as a marker of nuclear compartmentalization, the nucleolus performs several functions in cellular metabolism. The main function of the nucleolus, however, is as an rRNA synthesis site and involves ribosome assembly (Martin et al., 2015). Under an electron microscope, the nucleolus generally presents three major nucleolar components: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). However, during nucleologenesis, these compartments disorganize and reorganize, and in doing so, establish a nucleolar cycle (Zatsepina et al., 1997). There are many key proteins involved in maintaining nucleolar physiology. One of these proteins is fibrillarin, which is a small nuclear ribonucleoprotein (snRNP) that functions in methylation and in pre-rRNA processing. It associates with certain small nucleolar RNAs (snoRNAs) located in the nucleolar DFC, such as U3, U8, and U13 (Nicol et al., 2000). Fibrillarin expression has also been found to be essential for initial embryo development and cell growth, since fibrillarin (-/-) mouse models have been found to be unviable in early stages of development (Newton et al., 2003; Amin et al., 2007).

The presence of more than 10 microRNAs (miRNAs) was recently found in nucleolar compartments. Interestingly, these miRNAs were found to be present independently of Dicer activity or the main nucleolar enzyme (RNA pol I). Therefore, they are not associated with the transcription of ribosomal precursor RNA (Bai et al., 2014). Because the function of these miRNAs in the nucleolus is unknown, their presence may further link nucleolar physiology to CB physiology, especially given the fact that CB functioning is strongly based on existing microRNA pathways in the molecular composition of this organelle.

Some authors have determined that the CB originates from a bundle of intermitochondrial material present in the cytoplasm of germ cells (Fawcett et al., 1970; Reunov et al., 2000). However, this proximity to mitochondria may not be directly linked to the movement of these organelles toward the caudal region of spermatozoids, as proposed previously; instead, it may be associated with the fact that mitochondria participate in the miRNA pathway (Ernoult-Lange et al., 2012), which is frequently present in the CB.

Regardless of its origin, the activities of this cytoplasmic structure include the control of important processes for spermatogenesis: the presence of mutations in some components of the CB, such as TDR1/MTR-1, MVH and MIWI proteins and OX3 histocompatibility antigen, have been found to cause male sterility or a reduction in fertility in rats and mice (Head and Kresge 1985; Toyookaa et al., 2000; Deng and Lin 2002; Chuma et al., 2006). Due to the many questions that remain regarding the function of the CB and the evident importance of this structure to spermatogenesis, its characteristics need to be better understood in all classes of animals.

The aim of this study was to analyze the variation in fibrillarin nucleolar protein expression in mammalian epithelial germ cells in different stages of the spermatogenic cycle. This study also sought to determine whether this nucleolar protein is associated with the formation of CBs in initial spermatids. To analyze the relationship between fibrillarin and CBs, two markers of this cytoplasmic structure were used: the PIWIL1 protein (a member of MIWI proteins referred to herein as MIWI) and the HSP70 protein. These two markers exhibit different physiological functions: the MIWI protein, one of the most abundant components in the CB, plays a role in mRNA metabolism within this structure, while the HSP70 protein is a chaperone that aids in the degradation of protein components that do not have a function in the final stages of spermatogenesis. If fibrillarin is found to be integrated with some of these CB components, it could provide initial clues into the involvement of the nucleolar cycle in CB formation.

2. Materials and methods

In this study, 15 male Swiss mice (*Mus musculus*) were used. They were between 2 and 4 months of age and were supplied by the vivarium at Sagrado Coração University (USC) in São Paulo State, Brazil. During the experiments, the animals were cared for in accordance with the Brazilian Animal Welfare Regulations, or the DBCA (2013), which is monitored by the Brazilian Institutional Animal Care and Use Committee (CONCEA). The animals were kept in cages with food and water that was offered *ad libitum*, at a controlled temperature (between 21° and 25 °C), and with a 12:12-h light:dark cycle. For biological material collection, the animals were euthanized through the injection of barbiturates followed by cervical dislocation. All of the procedures used for euthanasia are consistent with the CONCEA Euthanasia Practice Guidelines (CONCEA 2013). This study was approved by the Ethics Committee for Animal Research and Education from the University of São Paulo, Bauru School of Dentistry (FOB-USP) under protocol No. 005/2013.

After the seminiferous tubules were obtained, they were separated by spermatogenic cycle stage, which was determined via observation under a transilluminating dissection microscope (Kotaja et al., 2004).

2.1. Immunofluorescence in squash preparations

The preparations were performed according to the protocol provided by Kotaja et al. (2004). The slides containing the squash preparations were immersed in a 4% PFA/PBS solution in ice for 10 min. Next, the slides were washed in PBS and then immersed in 0.2% Triton-X100/PBS at room temperature for 5 min. Non-specific staining was blocked using 5% BSA/PBS for 1 h at room temperature. After the blocking process, the samples were incubated overnight at 4 °C with the following primary antibodies: Anti-Fibrillarin [38F3] antibody ab4566 (Abcam, Sao Paulo, Brazil); PIWIL1 (N-17): sc-22685 (Santa Cruz Biotechnology, Sao Paulo, Brazil); and HSP 70 (K-20): sc-1060 (Santa Cruz Biotechnology, Sao Paulo, Brazil). After incubation, the slides were washed in PBST (PBS plus 5%Tween 20). The secondary antibodies of interest, which were conjugated with different fluorescent agents (Alexa Fluor® 488 F(ab')2 Fragment