Localization of Topoisomerase 2α and β During the Rat Spematogenesis Cycle

Lihua Liu[1], Ben Emery[1], John R. Gannon[1] and Douglas T. Carrell*[,1,2,3]

[1]*Andrology and IVF Laboratories, Division of Urology, Department of Surgery, School of Medicine, University of Utah, Salt Lake City, Utah 84108, USA;* [2]*Department of Obstetrics and Gynecology, School of Medicine, University of Utah, Salt Lake City, Utah 84108, USA;* [3]*Department of Physiology, School of Medicine, University of Utah, Salt Lake City, Utah 84108, USA.*

Abstract: Spermatogenesis involves the processes of germ cell proliferation, meiosis, and maturation. Germ cells at different spermatogenetic developmental stages exist through out the seminiferous tubules. In cross-sections of seminiferous tubules, staging of spermatogenesis is usually performed using PAS-hematoxylin staining, however, most studies also involve immunofluorescence analysis, which can be negatively affected by cell staining. Therefore, finding staging markers compatible with commonly used immunofluorescence techniques could be technically advantageous. In this study rat testis sections were evaluated for immunofluorescence expression profiles of topoisomerase 2α and topoisomerase 2β (Top2α and Top2β). Stage-specific patterns were seen for both proteins. Topoisomerase 2α was present in the nuclei of the spermatocytes from spermatogenesis stages 7 to 14. Alternatively, topoisomerase 2β was present in the nuclei of elongating and condensing spermatids. It was observed initially as 2-4 small foci in spermiogenesis step 2, then as a single focus near the nuclear membrane of round spermatids from spermiogenesis steps 3 to 8. The Top2β foci were distinct from the chromatoid body and proximal to the Golgi apparatus, which is known to be adjacent to the acrosome. Based on these specific findings we propose that the Top2β locates to the acrosome and that Top2α and Top2β immunofluorescence may be useful staging markers.

Keywords: Seminiferous epithelium, topoisomerase 2α, topoisomerase 2β, spermatogenesis, immunofluorescence.

INTRODUCTION

Spermatogenesis may functionally be divided into three phases, the proliferative phase, meiotic phase, and differentiation [1]. In the proliferative phase, spermatogonia undergo numerous cycles of mitosis to build a large population of cells that will subsequently develop into spermatocytes. In the meiotic phase, the spermatocytes undergo genetic recombination and reduction, producing haploid cells. During the meiotic phase, spermatocytes may be divided into; preleptotene, leptotene, zygotene, pachytene, diplotene, and secondary spermatocytes. After meiosis, the spermatids go through a series of differentiations or maturation, which includes nuclear condensation, spermatid head shaping, acrosome formation, and development of the flagellum. Because spermatogenesis takes place in synchronized waves, germs cells may be found in different phases throughout the seminferous tubules. Cell groupings or associations of germ cell types at specific developmental progressions are known as stages.

PAS-hematoxylin staining is the commonly used method for staging the cycle of seminiferous epithelium. One of the important criteria used to stage the cycle of seminiferous epithelium is the presence and the size of the round spermatid acrosome, which can be specifically identified by staining with PAS [1]. However, studies of spermatogenesis nearly always involve immunofluorescence techniques, which is negatively affected by the use PAS-hematoxylin stain. Therefore, finding spermatogenesis stage markers compatible with immunofluorescence microscopy is important for spermatogenetic study.

During spermiogenesis, sperm chromatin undergoes dramatic remodeling involving the relaxation of DNA supercoils and stepwise replacement of histones with transition proteins and protamines [2, 3]. This relaxation is facilitated by topoisomerases [4]. Topoisomerases have also been shown to be important in the chromatin remodeling in spermatogenesis [5, 6]. In this study, we found topoisomerase 2α and 2β were both present in germ cells in a stage-specific manner. Topoisomerase 2α and 2β may be used concurrently with immunofluorescence and therefore can be used as markers in staging seminiferous epithelium cycle.

MATERIALS AND METHODS:

Animal and Chemicals

Following institutional IACUC approval, testes of sexually mature Sprague-Dawley rats (Charles River, Wilmington, MA) were removed and immediately decapsulated and a portion of each was immediately fixed in Bouin's Fixative (Sigma Chemical Co., St Louis, MO) and stored at 4 °C until processed for histology. In order to characterize Top2α and Top2β in the cells of the rat

*Address correspondence to this author at the University of Utah Andrology and IVF Laboratories, 675 Arapeen Drive, Suite 205, Salt Lake City, UT, USA, 84108; Tel: +1-801-581-3740, Fax: +1-801-581-6127;
E-mail: douglas.carrell@hsc.utah.edu

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seminiferous tubules, 54 rat testis tissue sections from 27 animals, including both the left and the right testes were studied using immunofluorescence microscopy.

Antibodies and Other Chemicals

All primary antibodies were obtained from Abcam (Cambridge, MA). The rabbit polyclonal anti-topoisomerase 2α (ab45175) against the C-terminal of human Top2α and the rabbit polyclonal anti-topoisomerase 2β (ab58442) against amino acids 21-60 of the human form of Top2β were used. The rabbit polyclonal anti-DDX4 (ab13840) was used as a chromatoid body marker [7], and mouse monoclonal anti-GM130 (ab1299) was used as a marker for the Golgi apparatus. The Top2α neutralizing peptide (ab40178) was used as a negative control. Alexa fluor 594 or Fluorescein coupled, goat anti-rabbit, IgG (H+L) (A11012 and F2765) and Alexa fluor 488 coupled, goat anti-mouse, (H+L) (A11029) were obtained from Invitrogen (Carlsbad, CA). Normal rabbit IgG Santa Cruz Biotechnology (Santa Cruz, CA) was used as a negative control for Top2α and Top2β staining. Citrus clearing solvent was purchased from Thermo Fisher Scientific Inc., (Waltham, MA). All other chemicals were all obtained from Sigma Chemical, unless specifically noted.

Immunofluorescence Analysis

After fixation, the testicular tissue was embedded in paraffin and sectioned at 3 μm. Tissue was then mounted on Superfrost Plus glass slides (VWR, West Chester, PA 19380). Slide-mounted sections were de-waxed in citrus clearing solvent for 2-5 minutes. The sections were then rehydrated in gradually decreasing concentrations of ethanol (100%, 100%, 95%, and 80%), each for 5 minutes, then finally in running tap water for the final 5 minutes. Antigen retrieval was achieved by heating the slides in citrate-EDTA buffer (10 mM citric acid, 2 mM EDTA, 0.05% Tween-20, pH 6.5) at 120°C for 10 minutes, then immediately cooled in running tap water for 10 minutes.

Immunofluorescence analysis was performed following the manufacture's (Abcam) recommended protocol with minute changes. The slides were blocked in 10% goat serum TPBS (0.05% Tween-20 PBS) for 30 minutes. The working dilutions for anti-Top2α, anti-Top2β, anti-DDX4 (MVH), and anti-GM130 were 1:600, 1:300, 1:100, and 1:300, respectively. The working dilution for all secondary antibodies was 1:1000. The normal rabbit IgG and the Top2α neutralizing peptide were used in negative control experiments for anti-Top2β and anti-Top2α, respectively.

Staging of the Rat Seminiferous Epithelium Cycle

Following the immunofluorescence, PAS-hematoxylin was performed on all the testis sections to determine the stage of the seminiferous epithelium cycle. The rat seminiferous epithelium cycle was divided into 19 stages and the rat spermatids were further classified into 19 steps as described by Russell *et al.* [1]. Key criteria used in the staging included the following guidelines. Pachytene spermatocytes are present in the stages 12 to 14, with size gradually increasing. Secondary spermatocytes only exist in stage 14 tubules, while round spermatids (steps 1 to 8) and elongated spermatids (steps 15 to 19) are present in the seminiferous tubules stages 1 to 8. Elongating spermatids (steps 9 to 11) are present in the seminiferous tubules stages 9 to 11, while condensing spermatids (steps 12 to 13) are in the seminiferous tubules stages 12 to 13. The condensed spermatids (step14) are found in stage 14. Neither proacrosome nor acrosome exist in step 1 spermatids, but multiple proacrosomal granules and / or proacrosomal vesicles are present in step 2 spermatids, which fuse to form a single acrosome in later steps [1].

Microscopy and Imaging

Epifluorescence microscopy was performed using Zeiss Axioplan 2 Imaging System, and the images were taken with MetaSystems Isis imaging program (Baden-Württemberg, Germany). Micrographs of the PAS-hematoxylin stained seminiferous tubules were obtained using a Nikon camera mounted with a 20x objective lens.

RESULTS

Topoisomerase 2β Detection

Top2β was observed in the nuclei of the elongating and condensing spermatids (Fig. **1**) and in the cytoplasm near the nuclear membrane in the round spermatids as a single dot or rod-like structure (Fig. **1C**) , The same seminiferous tubules stained by PAS- hematoxylin following immunofluorescence are shown in Fig. (**1B**) and (**1D**). These patterns were not detected in the negative control. The chromatoid body, Golgi apparatus, and acrosome are all perinuclear in the round spermatids. The anti-DDX4 and anti-GM130 were used as markers of chromatoid body and Golgi apparatus, respectively. Topoisomerase was separate from the chromatoid body (Fig. **2A**), but was proximal to the Golgi apparatus, which is known to be adjacent to the acrosome (Fig. **2B**). Although the fluorescing stains were also ultimately observed in the residual body and midpiece of spermatids and mature sperm, they were nonspecific, as they appeared similarly in the negative controls.

Topoisomerase 2β was present in spermatids in a step-specific manner (Fig. **3** and Table **1**). No Top2β was present in the spermatids at step 1 but two to four small dots were observed in the cytoplasm of the spermatids by step 2. A single dot or rod was detected in the cytoplasm of the spermatids at steps 3 to 8. Top2β which, may appear as a dot initially, gradually became longer and more rod-like. Top2β expression was highest and localized to the cytoplasm in step7 spermatids. The Top2β ctyoplasmic expression in step 8 spermatids decreased or disappeared. However Top2β started to appear in the nuclei of step 9 spermatids with its intensity gradually increasing until the 12^{th} step. The signal became weaker following this and disappeared by step 14-spermatids. Showing a step specific localization of Top2β.

Topoisomerase 2α Detection

The Top2α was present in the nuclei of spermatocytes from stage 7 to stage 14. In the pachytene (stages 7 to 12) and diplotene (stage 13) spermatocytes, the fluorescence of Top2α appeared as bright foci encompassing the nucleus. In the secondary spermatocytes, it intensified around the metaphase chromosomes. It was also present in the nuclei of step 1 round spermatids (Fig. **4**). No intracellular fluorescence was observed in spermatocytes or spermatids in

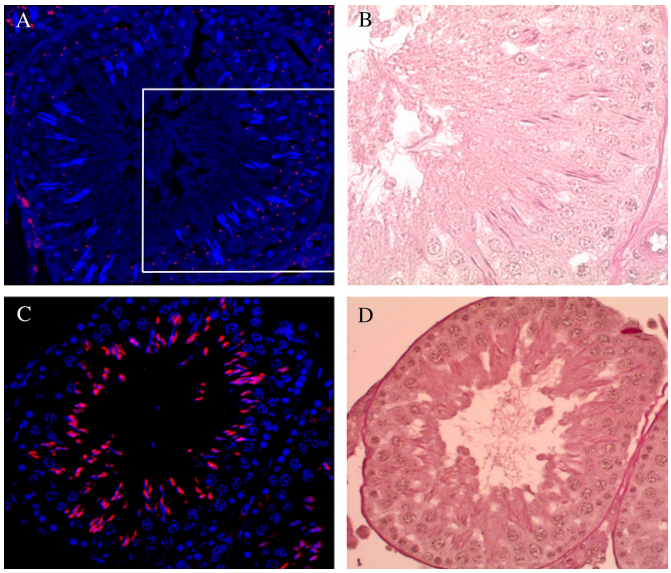

Fig. (1). Seminiferous tubule sections with immunofluorescence imaging of Top2β (panels A and C) and subsequently analyzed by PAS-hematoxylin staining on the same sections (panels B (square crop in A is the subset in B) and D). In panel A, Top2β (red) is found in the nuclei of the elongating spermatids of the stage XI tubule. In panel C, Top2β (red) is found in the round spermatid cytoplasm near nuclear membrane of the stage VII tubule.

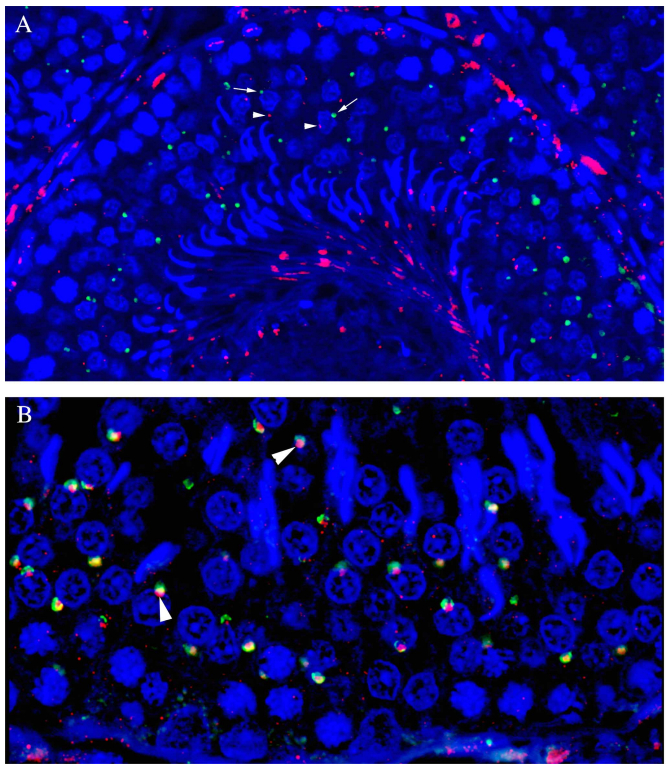

Fig. (2). Immunofluorescence labeling of Top2β, the chromatoid body, and the Golgi apparatus. A) Top2β (red, denoted by arrowhead) and chomatoid body (green, denoted by arrow) were both near the nuclear membrane, but were separate from each other. B) Top2β (Red, denoted by arrowhead) and Golgi apparatus (green).

the negative control experiments. This again showed the ability of Top2α to help differentiate germ cell stages.

DISCUSSION

Topoisomerase is a ubiquitous enzyme group, which is involved in a variety of activities including; DNA replication, transcription, recombination, and chromatin condensation. Although Top2α and Top2β share high homology in their primary sequences, they play different roles in cell biology. Top2α has previously been reported to be present in proliferating cells, while Top2β was predominantly seen in non-proliferating cells [8].

In our study, Top2α was observed in the nuclei of spermatocytes in seminiferous cycle stages 7 through 14, including pachytene, diplotene, and secondary spermatocytes. This observation is supported by two

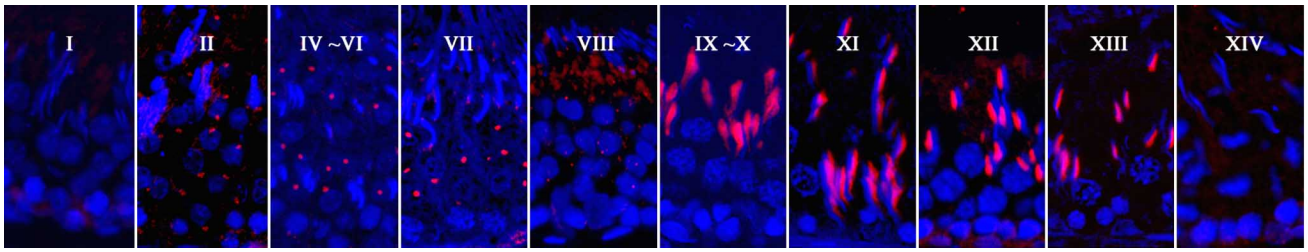

Fig. (3). Top2β (red) observed at the different stages of rat seminiferous tubes.

Table 1. The Localization of the Top2β in the Different Steps of Spermatids

Spermatid steps	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Acrosome	-	+	+	+	+	+	+	±	-	-	-	-	-	-	-	-	-	-	-
Nucleus	-	-	-	-	-	-	-	-	±	+	+	+	+	-	-	-	-	-	-

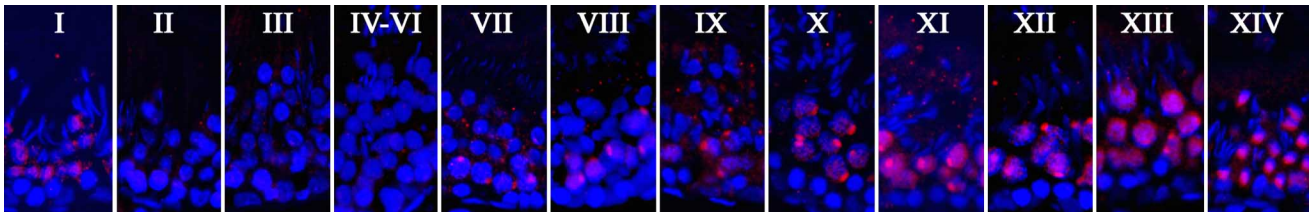

Fig. (4). Top2α (red) observed at the different stages of rat seminiferous tubes.

previously reported studies in mice, including a report by Cobb *et al.* in which Top2α was associated with the centromeric heterochromatin, which is involved with chromosome condensation, in the G2 phase of mitotic and meiotic cells [9]. Additionally, Rattner *et al.* reported that Top2α associated preferentially with centromeric heterochromatin in mitotic cells [10]. These observations support the hypothesis that Top2α likely plays an important role in chromosome condensation.

While Top2α is present in proliferating cells, the Beta isoform is predominantly present in non-proliferating cells like neurons and spermatids [5, 8]. This suggests Top2β has functions specifically related to cell differentiation and maturation [11]. In spermatids, chromatin condensation and DNA compacting are an important part of spermatid differentiation and mature sperm function. In our study [12, 13], Top2β was observed in the nuclei of the rat spermatids from steps 9 to 13, which is similar to the observation in mice made by Leduc and coworkers [5]. Due to its presence with chromatin remodeling in elongating and condensing spermatids, Top2β is believed to facilitate the chromatin condensation of spermatids.

In addition to its presence in the nuclei of the elongating and condensing spermatids, Top2β was detected inside the cytoplasm of all stages except stage 1 round spermatids in our study, giving it a very specific and localized function within the cell. Golgi apparatus, and acrosome are perinuclearly located. There is no acrosome in the spermatids at step 1, but two to four small proacrosomal vesicles appear in the spermatids at step 2. A single acrosome forms in the spermatids by step 3 and gradually lengthens with the progression of steps. By step 7 the acrosome covers one-third of the surface of the spermatid nucleus. The Golgi apparatus is involved in supplying materials for the formation of an acrosome. The Golgi positions itself near the acrosome to contribute more materials to the developing acrosome. By step 7, the Golgi apparatus starts to move away from the acrosome.

The chromatoid body, also perinuclear, contains RNA that may be important for synthetic processes at a later time. Toposisomerase 2β was observed as a single dot or rod in the cytoplasm near the nuclear membrane of step 3 to step 8 round spermatids, with the exception of two to four smaller dots inside the cytoplasm of step 2 round spermatids. The Top2β stain in round spermatids, that is clearly separate from the chromatoid body, was proximal to the Golgi apparatus until step 7, which is known to be adjacent to the acrosome. Therefore, we propose that Top2β is only located in the acrosome in round spermatids, not associated with the chromatoid body.

While the functions of Top2β still require further elucidation, the characteristic location of Top2α and Top2β can help stage the seminiferous epithelium cycle and identify the developmental stages of the germ cells. These data indicate that Topoisomerase 2α and 2β immunofluorescence may provide a viable alternative or support to PAS-hematoxylin staining to stage germ cell development within the seminiferous tubules. The interesting expression profile also suggests possible mechanistic and functional features of topoisomerase expression.

CAPSULE

The expression profiles of topoisomerase 2α and topoisomerase 2β can help in staging the cycle of the seminiferous epithelium during spermatogenesis.

ACKNOWLEDGEMENT

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CONFLICTS OF INTEREST

None Declared.

ABBREVIATIONS

Top2α = Topoisomerase 2α.

Top2β = Topoisomerase 2β.

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