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ORIGINAL ARTICLE

Gonocyte transformation in congenital undescended testes: what is the role of inhibin-B in cell death?

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Abstract

Purpose Undescended testes (UDT) are subjected to heat stress, which can disturb gonocyte transformation as well as apoptosis. This study aims to describe the apoptosis pathway occurring during minipuberty of children with unilateral (UDT), and to investigate the role of inhibin-B.

Methods Testicular biopsies at unilateral orchidopexy of 10 boys (6–9 months old) with normal inhibin-B ($n=5$) or low inhibin-B ($n=5$) were selected for immunohistochemistry and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay. Testicular tubules were labelled with antibodies against Anti-Müllerian hormone (AMH, Sertoli cell marker), mouse Vasa Homolog (MVH) and placental alkaline phosphatase (PLAP) (both germ cell markers), cleaved caspase3 (apoptotic marker), and followed by confocal imaging and cell counting with Fiji/ImageJ. Data were analyzed with GraphPad Prism.

Results In males with low and normal inhibin-B, there was no statistical difference ($p>0.05$) in the percentage of testicular tubules containing TUNEL + cells, number of cleaved caspase3 ± germ cells/tubule, total number of germ cells/tubule, and the percentage of fibrotic tubules or number of Sertoli cells/tubule.

Conclusions These results suggest that inhibin-B does not regulate cell death of gonocytes and further studies are required to uncover any role of inhibin-B in gonocyte transformation.

Keywords TUNEL · Caspase3 · Cryptorchidism · Inhibin-B · Gonocyte · Apoptosis

Introduction

Undescended testes (UDT) occur in up to 5% of neonatal males [1]. Mal-positioned testes located outside of the scrotum are subjected to heat stress, as the scrotum is a specialized low-temperature environment. Boys with persisting UDT beyond 6 months of age require early orchidopexy [2] to prevent malignancy or infertility after puberty [3]. However, the optimal time for this procedure is still unclear and knowledge about gonocyte transformation into spermatogonial stem cells is the key for understanding the correct time.

Gonocyte transformation is a complex process involving migration, differentiation, and proliferation or cell death [4], and occurs at minipuberty approximately 3–6 months of age [2]. Gonocytes begin in the centre of the seminiferous cords and transform into spermatogonial stem cells (SSC) when they reach the basement membrane. Impaired transformation of gonocytes leads to infertility [5].

"Minipuberty" describes a transient postnatal surge of gonadotrophin in humans which coincides with gonocyte

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transformation [5]. This gonadotrophin surge results in release of many hormones, including Inhibin-B [6], which is a paracrine hormone produced by Sertoli cells (SC), the support cells for the germ cells, and may be essential for the transformation of gonocytes into adult-dark spermatogonia [7]

This study aimed to assess which cell death pathways are involved during minipuberty of children with unilateral UDT using testicular biopsies and serum inhibin levels to investigate whether low inhibin-B has an impact on gonocyte transformation and apoptosis.

Materials and methods

Patients and specimens

Testicular biopsies were collected from 10 patients (6–9 months old) with unilateral UDT during orchidopexy in the Department of Paediatric Surgery, Copenhagen University Hospital Rigshospitalet, Denmark. At the time of orchidopexy, the serum levels of inhibin-B were measured: 5 patients with proven low serum inhibin-B (median 118 pg/ml, range of serum levels: 114–131 pg/ml; mean age = 8.2 months) and 5 patients who had normal serum inhibin-B (median 203 pg/ml, range of serum levels: 151– 75 pg/ml, mean age = 8.2) were selected for analysis. The biopsies were fixed in Stieve fixative and transported to the Murdoch Children's Research Institute, Australia followed by paraffin embedding (Ethics approval number 33139A).

TUNEL and immunohistochemistry

Sections of testicular biopsies (3microns) were de-waxed and microwaved for antigen retrieval in 0.01 M Citrate buffer (pH = 7.0), followed by blocking in 5% BSA (bovine serum albumin) plus 10% horse serum in PBS (phosphate-buffered saline) at RT for 2 h before TUNEL and antibody labelling. Primary antibodies for Anti-Müllerian hormone (AMH,), mouse Vasa Homolog (MVH, Abcam, AB13840,), placental alkaline phosphatase (PLAP), or cleaved caspase3 were incubated with the sections at 4 °C overnight followed by secondary antibodies (Table 1) and DAPI (4′6-Diamidino-2-phenylindole) staining as described previously [8].

A TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) is a means to label cells undergoing apoptosis with fragmented DNA. Two kits of TUNEL assays from two different companies (TUNEL1 from ThermoFisher Scientific, Catalogue# 10618; TUNEL2 from Roche, Catalogue#11 684 795 910) were used according to each manufacturer's recommendation. Each TUNEL assay was co-labelled with antibodies for AMH (Sertoli cell marker) and MVH (germ cells marker) followed by secondary antibodies. DNase treatment (1 µL/50 µL) was used as a positive control and reaction without enzyme as negative control for TUNEL. Undescended testis of an 8-week-old TS rat was also used as a positive control for TUNEL as there are apoptotic cells easily visible in this testis. Postnatal day-4 testis of a C57Bl/6 mouse was used as positive control for AMH and MVH antibodies as it has been confirmed by previous work of our group [8].

TUNEL assay combined with cleaved-Caspase 3 for co-localisation study

Four patients showing TUNEL^{+} cells in the testicular sections were examined for co-localisation of TUNEL and cleaved Caspase3^{+}. Briefly, tissue sections were incubated with cleaved-caspase 3 and AMH antibodies after TUNEL labelling.

Confocal imaging and data analysis

Fluorescent confocal images were obtained with a Zeiss LSM 780 confocal inverted microscope (Zeiss Microscopy Australia). The total number of germ cells, the number of germ cells on the basement membrane, and the number of Sertoli cells per cross-sectioned tubules were counted (82–125 testicular tubules/patient) using Fiji Image J (version 1.50, LOCI, University of Wisconsin-Madison,

Table 1 Primary and secondary antibodies used for Immunohistochemistry

Protein	Source and catalogue #	Species/isotope	Dilution for IHC
AMH	Santa Cruz, SC-6886	Goat	1/400
MVH	Abcam, AB13840	Rabbit	1/10,000
Cleaved-Caspase 3	Cell signaling, #9664	Rabbit	1/100
PLAP	BioGenex, MU228-UC	Mouse	1/400
Alexa-fluor 568	Invitrogen, A11057	Donkey anti-Goat	1/1000
Alexa-fluor 488	Invitrogen, A21206	Donkey anti-Rabbit	1/1000
Alexa-fluor 647	Invitrogen, A31571	Donkey anti-Mouse	1/1000
Alexa-fluor 568	Molecular probes, A11004	Donkey anti-Mouse	1/1000

Madison WI). Data were analysed with GraphPad Prism 7 using paired *t* tests.

Results

TUNEL assay

Most TUNEL$^+$ cells exhibited abnormal morphology with irregular antibody labelling which made it difficult to distinguish Sertoli cells from germ cells (Fig. 1). There was no statistical difference between the 2 TUNEL protocols (Fig. 2). Therefore, using the Roche® protocol, we found that there was no significant difference for the percent of testicular tubules containing TUNEL$^+$/AMH$^-$ (germ cells, $p=0.3101$) and TUNEL$^+$/AMH$^+$ cells (Sertoli cells, $p=0.62$) between the patients with low and normal serum inhibin-B levels.

In the low serum inhibin-B group, there was one patient with 1.22% of testicular tubules containing TUNEL$^+$ germ cells, while four other patients had no tubule containing TUNEL$^+$germ cells. In normal serum inhibin-B group, no patient had tubule containing TUNEL$^+$ germ cells (Fig. 2b). In low serum inhibin-B group, there were 3/5 patients with TUNEL$^+$ Sertoli cells (Fig. 3) in their testicular tubules (7.32%, 1.22%, and 6.48%), while in normal serum inhibin-B group, there were 4/5 patients with tubules containing TUNEL$^+$ Sertoli cells (3.26%, 0.86%, 3.51%, and 2.91%).

Cleaved caspase3

Cleaved caspase3 + was seen in the cytoplasm of both Sertoli cells (AMH$^+$) and germ cells (Fig. 4). There was heterogeneity in immunolabelling with varying intensity.

In patients with low inhibin-B, the average number of cleaved caspase3+ germ cells on the basement membrane per tubule was 1.48 (range 0.32–2.73), and in patients with normal inhibin-B, the average number of caspase3+ germ cells on the basement membrane per tubule was 1.47 (range 0.20–2.92), which was not a significant difference ($p=0.98$).

In patients with low inhibin-B, the average number of cleaved caspase3$^+$ germ cells off the basement membrane per tubule was 0.69 (range 0.03–1.61). In patients with the normal inhibin-B group, the average number of caspase3$^+$

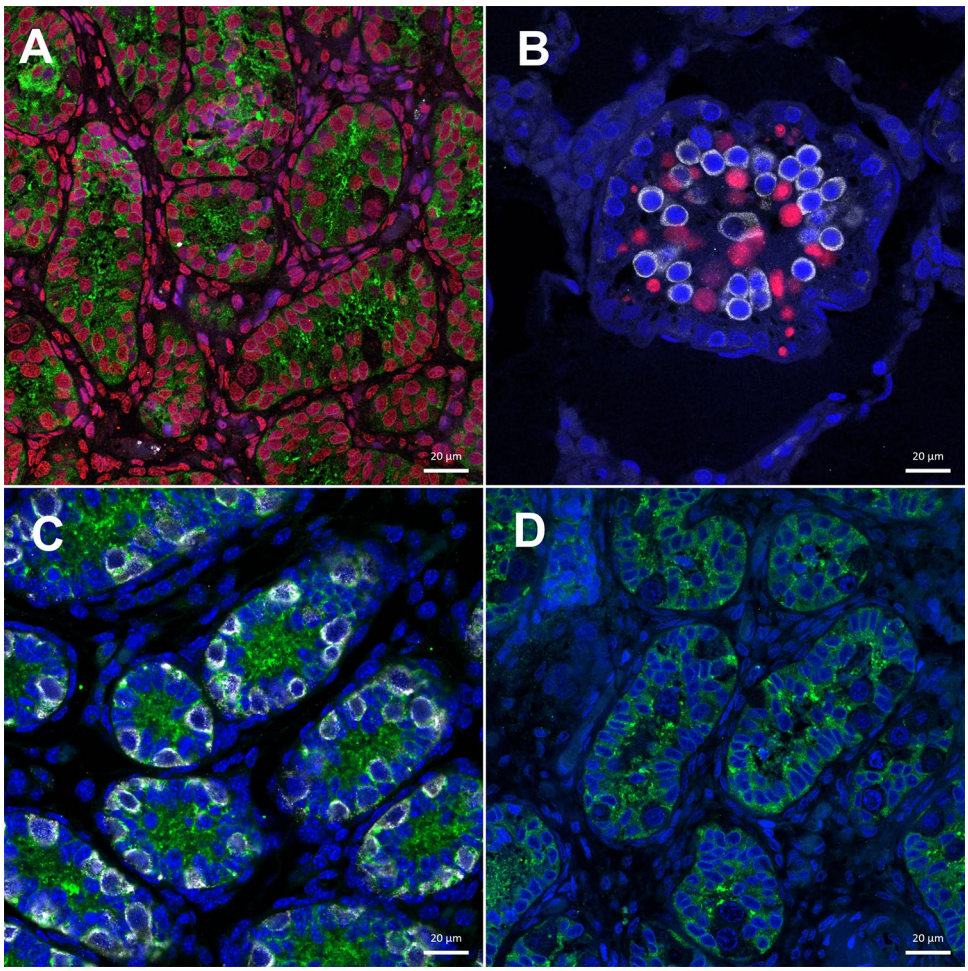

Fig. 1 Immunofluorescent confocal images for TUNEL control sections. **a** TUNEL positive control with DNase treatment of testicular biopsy of patient. **b** 8-week-old UDT of TS rat tissue, used as second positive control for TUNEL. **c** Day 4 testicular tissue of C57Bl/6 mouse used for positive immunohistochemistry control. **d** TUNEL negative control with testicular biopsy of patient. Red—TUNEL$^+$ nuclei (labeled fragmented DNA), Green—AMH (Sertoli cells), Blue = DAPI (labels all nuclei), and White—MVH (cytoplasm of some germ cells)

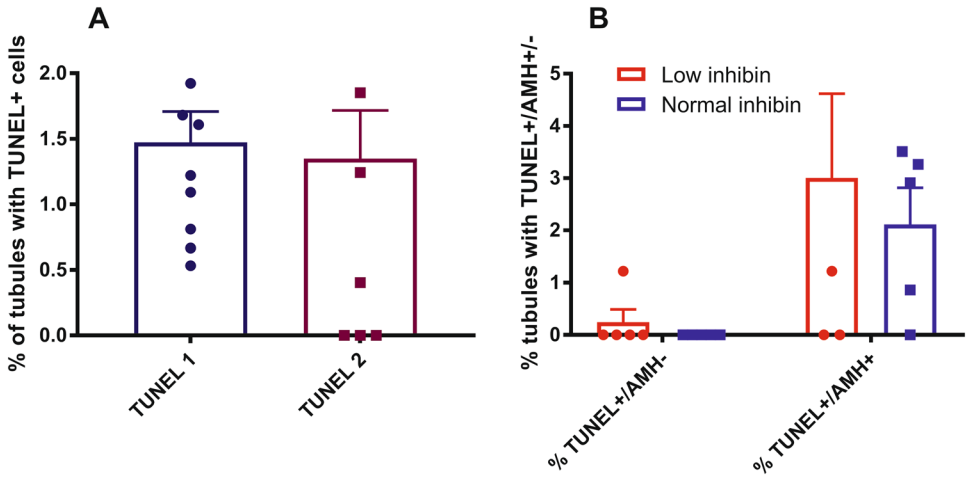

Fig. 2 Percent of testicular tubules containing TUNEL + cells. **a** Comparison of the percentage of testicular tubules containing TUNEL + cells between two TUNEL assay kits, Click-iT® Plus TUNEL assay (TUNEL1) and Roche® In situ Cell Death Detection (TUNEL2). **b** Using the TUNEL Roche® protocol, the percent of testicular tubules containing TUNEL + /AMH- and TUNEL + /AMH + cells

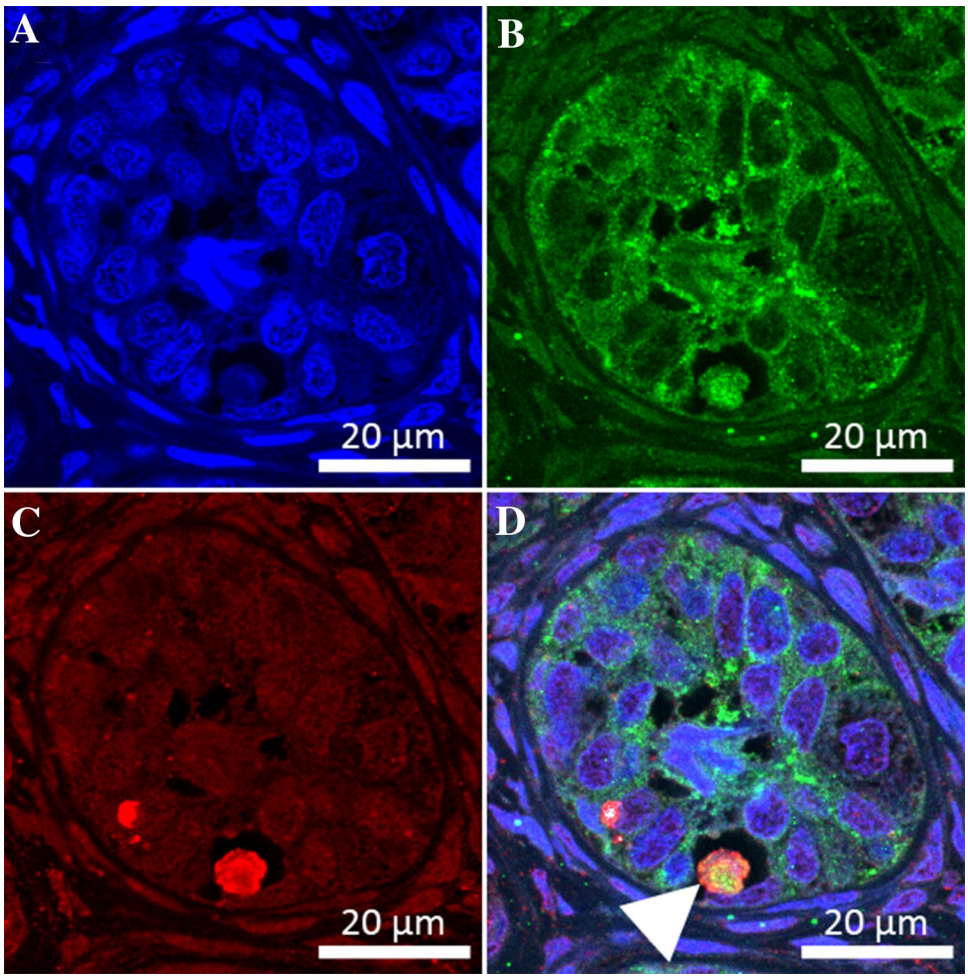

Fig. 3 Confocal images of testicular biopsies labelled with TUNEL assay. Testicular biopsy of patient labelled with **a** DAPI, **b** AMH, **c** TUNEL and **d** merged image of images **a**–**c** (arrowhead indicates a Tunnel + /AMH + cell, note AMH located in the nuclei)

germ cells off the basement membrane per tubule was 0.45 (range 0.01–0.79), which was also not statistically significant (Fig. 5).

As there was no statistically significant difference between patients with low or normal inhibin-B for cleaved caspase3 (positive or negative) germ cells (AMH^-) /tubule, the results from these two groups were pooled. There was significantly more caspase3$^+$ germ cells on basement membrane than off the basement membrane ($p = 0.019$) (Fig. 5). There was also a proportion of cleaved caspase3$^+$/AMH^+ putative Sertoli cells identified, but there was no

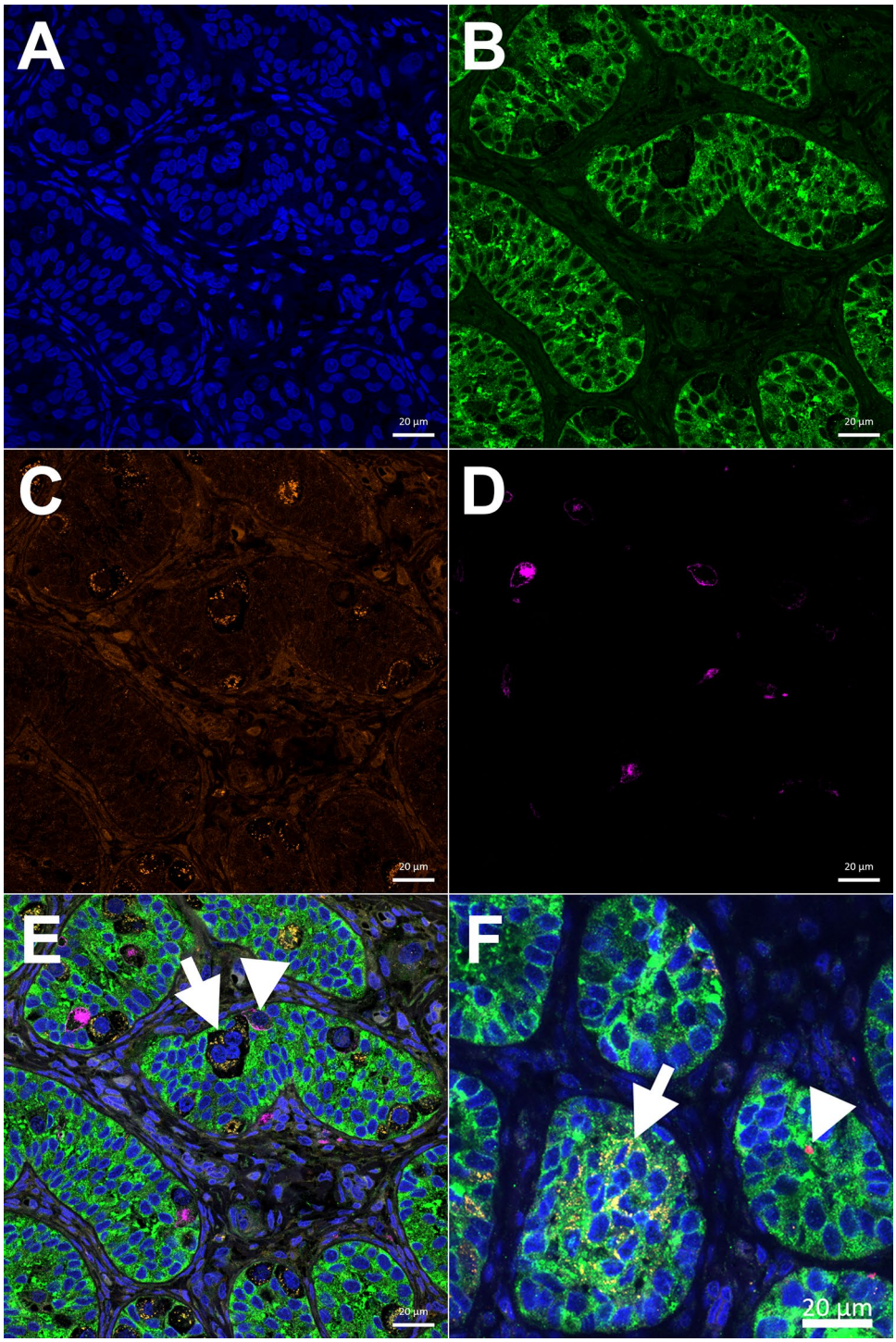

Fig. 4 Immunofluorescent confocal images of testicular biopsies. Testicular biopsy of patient labelled with **a** DAPI, **b** AMH, **c** cleaved Caspase3, and **d** PLAP. **e** Merged images **a**–**d** arrow indicates a cleaved Caspase3 + germ cell; arrowhead indicates a PLAP + germ cell. **f** TUNEL and cleaved caspase3 co-localisation study: arrow indicates fibrotic area of the tubule; arrow head indicates TUNEL + cell

significant difference between patients with normal and low serum inhibin-B.

TUNEL and cleaved caspase3 co-localisation study

The four patients with TUNEL + cells were analysed with immunolabelling for cleaved caspase3 to investigate whether labelling for TUNEL and cleaved caspase3 occurred in the same cell. Co-localisation was not observed in any of the four testicular biopsies investigated.

Total germ cells and total Sertoli cells per tubule

Germ cells (AMH^-) could be adequately identified in testicular tubules of all patients; however, MVH and PLAP were also utilized to assist in identification. MVH and

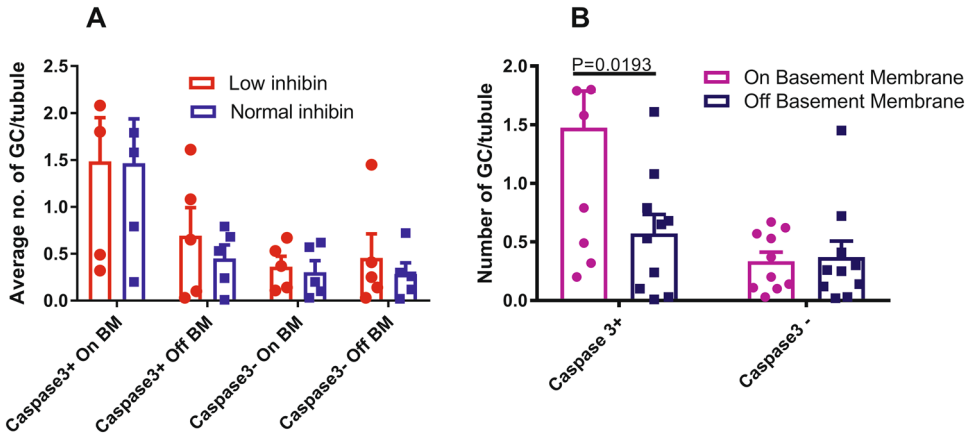

Fig. 5 Quantity of cleaved Caspase3 ± germ cells/tubule from testicular biopsies. **a** The quantity of germ cells labelling for cleaved caspase3 ± located on or off the basement membrane (BM) of testicular tubule. **b** Pooled data for cleaved caspase3 positive or negative-germ cells and their location (on or off BM of the testicular tubule). There was a statistically significant difference between the number of cleaved caspase3 + germ cells per tubule located on and off the basement membrane ($p = 0.0193$)

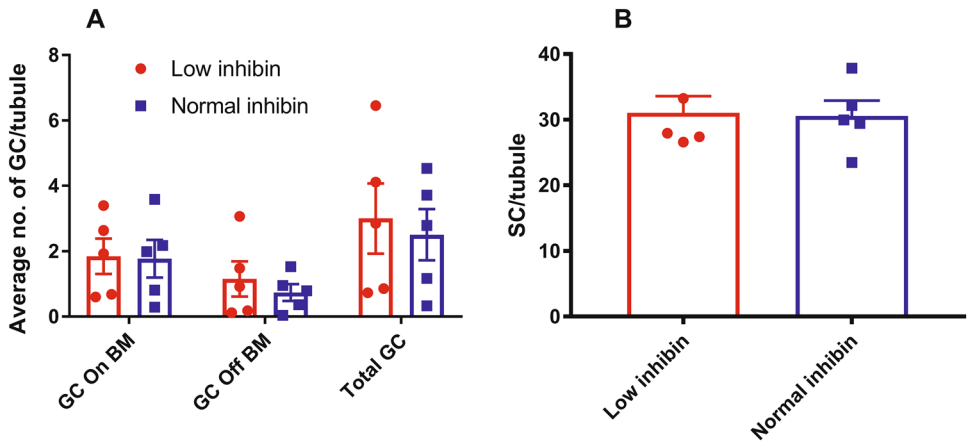

Fig. 6 Quantity of germ cells and Sertoli cells per tubule. **a** Total number of germ cells/tubule and their location in the testicular tubule (on or off the basement membrane) in patients with low and normal serum inhibin-B. **b** Number of Sertoli cells (AMH +)/tubule

PLAP immunolabelled a small proportion of the germ cells. There was no significant difference for total germ cells per tubule, nor Sertoli cells (AMH^+) per tubule between the two groups of patients with low or normal serum inhibin (Fig. 6).

Fibrotic tubules

Testicular tubules with clustered fibrotic cells were frequently observed and tubules with fibrosis were quantified based on the degree of fibrosis (Table 2). A tubule was considered fibrotic if there were greater than 1 densely DAPI-stained, dysmorphic nuclei clustered together. The average percentage of tubules with fibrosis in patients with low serum inhibin-B was 32.37% and that in patients with normal serum inhibin-B was 24.23%, with no statistical significance.

Table 2 Degree of fibrosis and percentage of fibrotic testicular tubules

Degree of fibrosis	Average % fibrotic tubules for low serum inhibin-B group	Average % fibrotic tubules for normal serum inhibin-B
+[a]	17.69	18.16
+ +[b]	27.22	17.74
+ + +[c]	55.09	64.10

[a] = 2–5 dense, clustered nuclei

[b] = 6–10 dense, clustered nuclei

[c] = > 10 dense, clustered nuclei

Discussion

Some germ cells immunolabelled for cleaved caspase3, indicating that these cells were undergoing caspase3-mediated apoptosis, but there was no statistically significant

difference between the two groups of patients with low or normal serum inhibin-B. There was also no significant difference between these two groups of patients in regards to the number of germ cells/tubule, whether they were located on the basement membrane (likely spermatogonial stem cells, SSC) or not (off tubular basement membrane), which are considered to be gonocytes, and the number of Sertoli cells/tubule, as well as percentage of tubules with clusters of fibrotic cells which are degenerating cells. This indicates that serum inhibin-B levels at the time of orchidopexy may not play an important role in the gonocyte transformation into SSC.

Using TUNEL assay, a marker of advanced apoptosis [9], we found that it was more common for patients to have tubules containing $TUNEL^+$ Sertoli cells than $TUNEL^+$ germ cells. This suggests that Sertoli cells during or just after minipuberty may be more susceptible to apoptosis than germ cells. However, most $TUNEL^+$ cells exhibited abnormal morphology with irregular antibody staining, which made it difficult to distinguish Sertoli cells from germ cells (Fig. 4).

There was heterogeneity in caspase3 staining as some cells had more intensity and others with less intensity, which suggests that cells were at a different stage of the apoptotic process but with no difference between the number and proportion of the germ cells expressing cleaved $caspase3^+$ in patients with the low and normal serum inhibin-B groups. This suggests that the apoptosis occurring may not be under control of inhibin-B. Culty et al. describe cell death to be an intrinsic part of gonocyte transformation [4] and our results support this. Cell death may be initiated after the gonocytes reach the basement membrane (and hence become SSC), and the majority of cells that undergo apoptosis do so during minipuberty. The patients involved in our study were slightly older, (6–9 months old) just passed the minipuberty period. This may also indicate that apoptosis may be slightly delayed in UDT or apoptosis is a progressive process during testis development to eliminate any unfit gonocytes and SSC.

Unexpectedly, there was great disparity between the labelling of $TUNEL^+$ nuclei and cleaved $caspase3^+$ cells, and in our co-localisation study for both TUNEL and $caspase3^+$, there were no cells staining for both TUNEL and cleaved caspase3. However, this could also indicate that the $caspase3^+$ cells are in the process of apoptosis and have not yet undergone fragmentation of their DNA, but may have a higher frequency at a more advanced age.

The gonocyte has been described as the "forgotten cell of the germ line" [10]. In our study, we found no differences between patients with low and normal serum inhibin-B in the total number of germ cells that had already reached the basement membrane and completed transformation and differentiated into SSC. Andersson et al. state that in pre-pubertal males, the levels of serum inhibin-B were independent of the number of GC [11]. Our results reflect this, and may suggest that inhibin-B does not play a major role in migration, proliferation, or transformation into SSC.

Sertoli cells are the support cells for gonocytes and thus play an important role in gonocyte transformation [12], but there was no statistically significant difference between the two groups of patients with low and normal serum inhibin-B. Cortes et al. state that inhibin-B levels may be reflective of the histological state of seminiferous tubules, however, also report that levels of inhibin-B in boys with UDT have yielded mixed results [7, 13]. In addition to this, Maechem et al. also postulate that inhibin-B levels most likely reflect proliferation of Sertoli cells [14]. As there was no difference in either the number of Sertoli cells or germ cells in our study, regardless of low or normal serum inhibin-B, our results do not support inhibin-B being reflective of the histological state of seminiferous tubules. However, it may be due to a weakness of the study that the low group is just under the 2% percentile and all patients of the "normal" inhibin group were actually under the median [15]. Therefore, further studies are required to confirm our results.

A number of tubules had packed areas of dense, dysmorphic nuclei both in the centre and peripherally, as well as varying degrees of fibrosis. We also noted that there appeared to be autofluorescence in some of these degenerating tubules, coinciding with the observations of Li et al. [8].

Inhibin-B is a paracrine hormone released by Sertoli cells [14]. During minipuberty, there is a transient surge of reproductive hormones acting on the testes, coinciding with gonocyte transformation, and there are still many questions about the role of its hormonal regulation. In the literature, gonocyte proliferation and migration is reported to occur independently of inhibin-B [11]. However, there is little known about whether inhibin-B has an impact on the number of germ cells or Sertoli cells undergoing cell death [16]. Our results suggest that inhibin-B does not appear to have a direct role in these processes. However, there is still more work to be done as approximately 10% of post-pubertal males with surgically corrected unilateral UDT will not achieve paternity [15] and there is evidence that low levels of inhibin-B in pre-pubertal cryptorchid males may predict this [13]. If a more in-depth understanding of the role of inhibin-B in gonocyte transformation is achieved, this could assist in optimizing long- and short-term management of these children.

There are several limitations of this study. There was a small number of samples, due to ethical limitations in acquiring testicular biopsies during orchidopexy. These ethical limitations also extend to comparing these biopsies to descended testes; however, as the focus of this question is on UDT and inhibin-B, this is not a huge problem. Immunohistochemical analysis has a risk of non-standardised cell identification as fluorescence intensity may be variable.

Availability of suitable antibodies for co-localization analysis also limits the precise measurement of cell types. In addition, labelling non-target cells may occur due to passive uptake or extracellular secretion of the target protein. TUNEL staining may also provide false positives [9], and hence, we used two markers for cell death. However, the benefits of this study are that we included many positive and negative controls to ensure that the method was working correctly. The two groups had the same age mix and all ages occurred just after minipuberty.

In conclusion, these results could suggest that inhibin-B does not play an important role in the regulation of cell death during gonocyte transformation or germ cell development at the postnatal age of 6–9 months. Further studies are required to confirm our results and uncover the role of inhibin-B in gonocyte transformation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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