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ORIGINAL ARTICLE

Male Reproduction

Postnatal germ cell development in cryptorchid boys

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Cryptorchidism is associated with infertility in adulthood. Early orchiopexy is suggested to reduce the risk. Information is lacking on the potential link between infant germ cell maturation and the risk of future infertility. The objective of the study was to evaluate age-related germ cell development in cryptorchidism. Immunostaining for markers of germ cell development (octamer-binding transcription factor 3/4 [OCT3/4], placental alkaline phosphatase [PLAP], KIT proto-oncogene [C-KIT], podoplanin [D2-40], Lin-28 homolog A [LIN28], and G antigen 7 [GAGE-7]) was performed in testicular biopsies from 40 cryptorchid boys aged 4–35 months. Germ cell numbers and distributions were evaluated in cross sections of seminiferous tubules, with and without immunostaining. *OCT3/4, D2-40*, and *LIN28* were generally expressed in the early stages of germ cell development, as shown by positive expression in germ cells in the central region of seminiferous tubules. In contrast, *PLAP* and *GAGE-7* were expressed in both central and peripheral parts of the tubules in the early stages of development and expressed mainly in a peripheral position with advancing age. Germ cell maturation was delayed in this study population as compared with that observed in our previous study on germ cell markers in a healthy population. The number of GAGE-7-positive germ cells per tubular cross section obtained by immunostaining was significantly higher than that obtained by standard hematoxylin and eosin staining. Double immunostaining revealed heterogeneity in germ cell development in cryptorchid testes. These results shed light on the pathophysiology of germ cell development in boys with cryptorchidism.

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INTRODUCTION

Around 1%–3% of boys in the Western world undergo surgery for cryptorchidism.[1–3] Cryptorchidism is associated with infertility in adulthood. Information is lacking on the potential link between infant germ cell maturation and the risk of future infertility.

In normal testicular development, gonocytes migrate from a central position in seminiferous tubules to a peripheral position on the basal membrane and transform to spermatogonia during the first 6 months of life.[4] The number of spermatogonial cells and the efficacy of gonocytes which transform into type A dark (Ad) spermatogonia are considered critical for future fertility.[5–8] Cryptorchidism is associated with the persistence of gonocytes after the age of 6 months and a reduced number of Ad spermatogonia.[5–7]

To evaluate germ cell development in the testes of cryptorchid boys, we routinely perform a histological examination of testicular biopsies at the time of surgery for cryptorchidism in our department.[9] Identification of all germ cells using standard hematoxylin and eosin (H&E) staining is difficult, and several intratubular germ cell neoplasia markers, such as cluster of differentiation 99 (CD99), podoplanin (D2-40), KIT proto-oncogene (C-KIT), octamer-binding transcription factor 3/4 (OCT3/4), and placental alkaline phosphatase (PLAP), have been applied to examine germ cell development using immunohistochemistry (IHC).[10–13] Thymocyte differentiation antigen 1, DEAD-box helicase 4, and stage-specific embryonic antigen-4 have also been suggested as markers of germ cells.[14] Based on RNA-sequencing, a previous study suggested that double sex and mab-3 related transcription factor like family C2 (DMRTC2), paired box 7 (PAX7), T-box transcription factor Brachyury, and telomerase reverse transcriptase were important for the transformation of gonocytes into Ad spermatogonia during mini-puberty.[15] However, the ability of IHC, either alone or in combination with other methods, to detect markers of germ cell development is unclear. As techniques for cryopreservation of testicular tissue from young boys, including those with cryptorchidism, begin to be implemented with the aim of ensuring fertility in adult life,[16] improved IHC markers of germ cell development are increasingly important in the evaluation of testicular biopsies from cryptorchid boys.

In a previous study, we focused on D2-40, C-KIT, OCT3/4, and PLAP, which are IHC markers routinely used for investigating testicular neoplasms and cryptorchid testes.[13] In this study, we also analyzed the expression of lin-28 homolog A (LIN28) and G antigen 7 (GAGE-7). LIN28 is a pluripotent stem cell regulator,[17] which is expressed by primordial germ cells, premeiotic germ cells in human fetal ovary, and gonocytes in fetal testis.[18–20] Aeckerle *et al.*[19] reported that LIN28 was present in fetal gonocytes and spermatogonia in the postnatal testis. GAGE-7 is a member of a family of cancer/testicular

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antigens, the expression of which is restricted to human malignancies and testes.[21] To our knowledge, these molecular markers have not been applied previously in studies of undescended testes to analyze germ cell development in a clinical setting.

The aim of this study was to evaluate age-related germ cell development in boys with cryptorchidism with immunofluorescent (IF) staining using a number of established new markers associated with different stages of germ cell development.

MATERIALS AND METHODS

Cryptorchid patients

This study included 40 boys with cryptorchidism (median age: 13 months, range: 4–35 months) who underwent orchiopexy between June 2015 and June 2017. Testicular biopsies were obtained at the time of the surgeries. The present study included biopsies from 20 boys from our previous study.[13] The same surgeon (JT) performed all the operations. Of the 40 boys, there were 29 cases of unilateral cryptorchidism and 11 cases of bilateral cryptorchidism. In total, 47 testes biopsies were obtained, as a biopsy was obtained from both the testes of seven of the bilateral cryptorchid patients. In all cases, the biopsies were obtained from a similar area of the testis. The uni- and bilateral biopsies were pooled for analysis. Patients with chromosomal abnormalities, associated anomalies, previous inguinal surgery, or hormonal therapy prior to surgery were excluded.

Tissue preparation and staining

Tissue samples were fixed in an Stieve's fixative (Solution I: 90 g $HgCl_2$ [Sigma, St. Louis, MO, USA] in 1.5 l of H_2O; Solution II: 400 g 40% formaldehyde [Struers KEBO lab, Albertslund, Denmark] and 80 g of 98% acetic acid [Sigma]). Just before use, 38 ml of Solution I was mixed with 12 ml of Solution II. After fixation, the tissue samples were dehydrated and embedded in paraffin. They were then cut into 2-μm sections and mounted on coated slides (Dako, Glostrup, Denmark). A section was stained with H&E. For IHC staining, all sections were incubated with primary antibodies CD99 (1:100, 12E7, Dako), D2-40 (1:25, M3619, Dako), C-KIT/CD117 (1:50, Dako), OCT3/4 (Cell-Marque Corp, Rocklin, CA, USA), and PLAP (1:200, PL8-F6, Biogenex, Fremont, CA, USA). IHC staining was performed on a Ventana Benchmark Ultra Stainer (Roche Diagnostics, Indianapolis, IN, USA).

IF staining was done manually. All sections were deparaffinized, subjected to antigen retrieval treatment using TEG buffer (10 mmol l^{-1} Tris [Sigma] and 0.5 mmol l^{-1} EGTA [Sigma], pH 9), and blocked for 0.5–1 h at room temperature in 1% BSA (Merck, Billerica, MA, USA) in TBS buffer (50 mmol l^{-1} Tris, 150 mmol l^{-1} NaCl, pH 7.6) before the primary antibodies were applied. The following polyclonal antibodies were used: a polyclonal rabbit anti-LIN28 antibody (diluted 1:200; Ab46020, Abcam, Cambridge, UK), mouse monoclonal anti-GAGE-7 antibody (diluted 1:100; G13520, Transduction Lab, Franklin Lakes, NJ, USA), polyclonal rabbit anti-C-KIT/CD117 (1:50, Dako), and mouse monoclonal anti-PLAP (1:100, PL8-F6, Biogenex). The slides were washed three times. The sections were stained with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse or anti-rabbit IgG antibody (diluted 1:500; Jackson ImmunoResearch, Skanderborg, Denmark) or Alexa Fluor 594 donkey anti-rabbit or mouse IgG antibody (diluted 1:500; Jackson ImmunoResearch) for 1 h at room temperature. The nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI) staining before mounting the slides with ProLong Gold Antifade Mountant (Life Technology, Eugene, OR, USA).

In addition, the sections were co-immunostained with different combinations of antibodies to evaluate codistribution of different markers. For biopsies of undescended testes from patients aged 6, 12, 18, and 24 months, dual IF staining with LIN28 and GAGE-7 was performed to study the kinetics of germ cell development. In addition, dual IF or IHC staining with C-KIT and PLAP, PLAP and LIN28, and C-KIT and GAGE-7 was performed in a testicular biopsy of a 10-month-old cryptorchid boy to investigate potential differences in germ cell staining between dual IF and IHC. Sections treated with blocking buffer (without primary antibody) served as negative controls.

Histological examination

The sections stained with H&E and the IHC markers (CD99, D2-40, C-KIT, OCT3/4, and PLAP) were examined in cross sections of seminiferous tubules. The total germ cell number per tubular cross section (G/T) and the number of specific type Ad spermatogonia (Ad/T) were counted in at least 100 seminiferous cross-sectional tubules per biopsy, as previously described.[22,23] Ad spermatogonia fulfilled the following criteria: (1) located along the basement membrane; (2) the presence of a rarefaction zone, centrally located within the nucleus; and (3) homogeneous deep staining of the nucleus, as previously described.[8,23–25] The normal value of G/T was based on findings recently published by our laboratory based on an analysis of forensic material.[26] The fertility index (FI%) was also measured. The FI% was the percentage of tubule transverse sections containing germ cells in at least 100 seminiferous cross-sectional tubules per biopsy, as previously described.[22,23] In IF staining, the number of LIN28- and GAGE-7-positive cells in at least 50 cross sections of the tubules was examined, and the G/T value and FI% were calculated. In cases of bilateral cryptorchidism, the mean values of G/T, Ad/T, and FI% from both testicular biopsies were calculated. To ensure that the number of fields counted were sufficient to represent the number of germ cells, at least 25 cross-sectioned tubules were included.[27]

In addition, for each age group of 4 months (4–8 months, 8–12 months, *etc*., up to 32–36 months), we calculated the total number of germ cells that were positive for each specific marker and the percentage of germ cell-positive staining for each marker. These figures were compared with corresponding values for D2-40, C-KIT, OCT3/4, and PLAP based on an analysis of forensic medicine material by our laboratory.[26] In addition, we classified the distribution of germ cells positive for the molecular markers according to their location in the tubules: only central, mainly central, equally central and peripheral, mainly peripheral, and only peripheral. The tubular distributions of the germ cells were defined according to their location. For example, all germ cells adherent to the tubular basement membrane were considered only peripheral.

Statistical analyses

The Mann–Whitney U test, Chi-square test, Fisher's exact test, linear regression, and logistic regression test were used and performed in Prism Graph program version 7 (GraphPad, San Diego, CA, USA).

Ethical approval

The study was conducted according to the Helsinki II declaration, and informed consent was obtained from the parents of the patients. The study received approval from the Ethics Committee of Copenhagen University Hospital of Rigshospitalet, Copenhagen, Denmark (KF-01299830).

RESULTS

Positive immunostaining in relation to age and tubular position

Table 1 and **Supplementary Table 1** provide data on the semiquantitative scoring of the positive immunostaining of germ

cells in relation to the patient's age and distribution of germ cells expressing the various markers (D2-40, OCT3/4, C-KIT, PLAP, LIN28, and GAGE-7) as well as data on Ad spermatogonia values in the testes of the 40 cryptorchid boys (28% bilateral). Germ cells positive for D2-40 were present up to the age of 12 months, whereas germ cells positive for C-KIT or OCT3/4 were detected up to the age of 16 months. The LIN28 signal in germ cells was present up to the age of 20 months. Germ cells positive for PLAP and GAGE-7 were detected in all age groups (**Table 1**). Germ cells positive for OCT3/4 or C-KIT were mainly located centrally. Those positive for D2-40 or LIN28 were equally distributed centrally and peripherally, and germ cells positive for PLAP or GAGE-7 were found mainly in peripheral locations in seminiferous tubules (**Supplementary Table 1**). Analysis of the relative number of germ cells positive for the different markers in three locations (mainly peripheral, equally distributed central and peripheral, and mainly central) revealed significant differences in positive staining for D2-40, C-KIT OCT3/4, PLAP, LIN28, and GAGE-7 (all $P < 0.0001$). No germ cells were positive for a marker only in a central or peripheral location. Representative images of H&E and IHC staining using the molecular markers (CD99, D2-40, C-KIT, OCT3/4, and PLAP) are shown in **Figure 1**. CD99 stained Sertoli cells and interstitial cells, and consequently, negative cells within tubules were counted as germ cells. D2-40 stained Sertoli cells, as well as germ cells. OCT3/4, C-KIT, and PLAP signals were present only in germ cells. PLAP germ cell expression was detected in all ages.

The results of dual IF staining of LIN28 and GAGE-7 in biopsies from undescended testes of boys aged 6, 12, 18, and 24 months are shown in **Figure 2**. The LIN28 signal mainly appeared in germ cells at a younger age. Interestingly, the GAGE-7 signal was found in all samples, irrespective of age. Germ cells positive for GAGE-7 were located both centrally and peripherally in seminiferous tubules during the 1st year

Table 1: Results of positive immunostaining of germ cells including Ad spermatogonia related to age in cryptorchid boys

Age (month)	*Total (n)*	*Ad spermatogonia, % (n)*	*D2-40, % (n)*	*C-KIT, % (n)*	*OCT3/4, % (n)*	*PLAP, % (n)*	*LIN28, % (n)*	*GAGE-7, % (n)*	*P**
4≤ age <8	9	67 (6/9)	78 (7/9)	100 (9/9)	100 (9/9)	100 (9/9)	100 (9/9)	100 (9/9)	0.065
8≤ age <12	9	89 (8/9)	22 (2/9)	56 (5/9)	56 (5/9)	100 (9/9)	78 (7/9)	100 (9/9)	0.0019
12≤ age <16	5	40 (2/5)	0	40 (2/5)	20 (1/5)	100 (5/5)	40 (2/5)	100 (5/5)	0.0041
16≤ age <20	11	45 (5/11)	0	0	0	100 (11/11)	9 (1/11)	100 (11/11)	<0.0001
20≤ age <24	2	0	0	0	0	50 (1/2)	0	100 (2/2)	0.095
24≤ age <28	3	0	0	0	0	67 (2/3)	0	67 (2/3)	0.068
32≤ age <36	1	0	0	0	0	100 (1/1)	0	100 (1/1)	0.3062
Total	40	53 (21/40)	23 (9/40)	40 (16/40)	38 (15/40)	95 (38/40)	48 (19/40)	98 (39/40)	<0.0001

*Chi-square test. Ad: type A dark; D2-40: podoplanin; C-KIT: KIT proto-oncogene; OCT3/4: octamer-binding transcription factor 3/4; PLAP: placental alkaline phosphatase; LIN28: LIN-28 homolog A; GAGE-7: G antigen 7

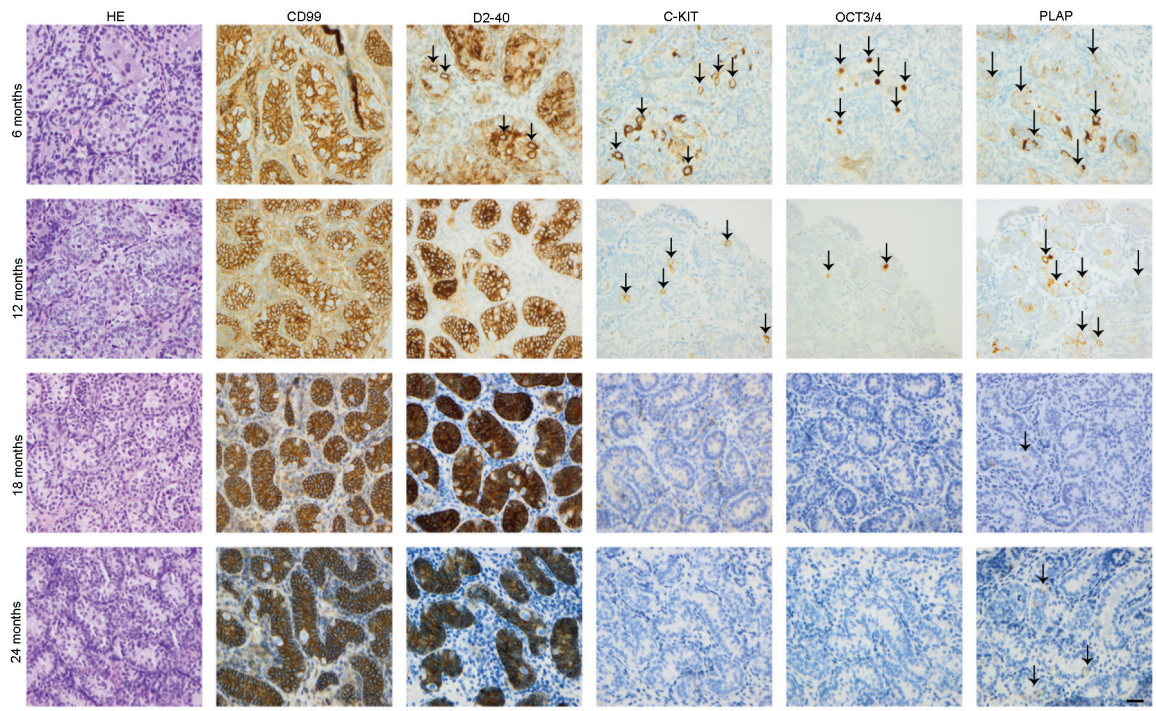

Figure 1: H&E staining and immunohistochemistry by CD99, D2-40, C-KIT, OCT3/4, and PLAP in undescended testis aged 6, 12, 18, and 24 months, respectively. Arrows show the positive signals. Scale bars = 20 μm. CD99: cluster of differentiation 99; D2-40: podoplanin; C-KIT: KIT proto-oncogene; OCT3/4: octamer-binding transcription factor 3/4; PLAP: placental alkaline phosphatase; LIN28: LIN-28 homolog A; GAGE-7: G antigen 7; H&E: hematoxylin and eosin.

of life, moving to a more peripheral location with age. Costaining of LIN28 and GAGE-7 showed that they were essentially different subpopulations (**Figure 2**). Dual immunostaining on a testicular specimen from a 10-month-old cryptorchid boy revealed that C-KIT and PLAP were partially overlapped (**Figure 3a**). *PLAP* was expressed in the subpopulation of LIN28-positive germ cells (**Figure 3b**). C-KIT and GAGE-7 represented different germ cell population (**Figure 3c**). The negative controls for IF are presented in **Supplementary Figure 1**.

The number of germ cells

When the results of H&E and IHC staining were combined, the FI% decreased with increasing age at the time of orchiopexy ($\beta = -1.459$, $P = 0.0003$; **Figure 4a**). The G/T also decreased with increasing age at the time of orchiopexy ($\beta = -0.052$, $P < 0.0001$; **Figure 4b**). In one boy aged 25 months, only Sertoli cells were detected, with no expression of the germ cell markers. The Ad/T also decreased with increasing age at the time of orchiopexy ($P = 0.0085$; **Figure 4c**).

As shown by the results of IF staining, the number of LIN28-positive germ cells per tubular cross section (G/T) decreased with increasing age at the time of orchiopexy ($\beta = -0.0353$, $P = 0.0023$; **Figure 4d**). Moreover, the percentage of tubular cross sections containing LIN28-positive germ cells also decreased with increasing age at the time of orchiopexy ($\beta = -1.145$, $P = 0.0004$; **Figure 4e**). Similarly, the percentage of tubular cross sections with GAGE-7-positive germ cells decreased with increasing age at the time of orchiopexy ($\beta = -1.356$, $P = 0.0009$; **Figure 4f**), and the number of GAGE-7-positive germ cells per tubular cross section decreased with age ($\beta = -0.061$, $P < 0.0001$; **Figure 4g**).

When the results of IF staining for GAGE-7 comparing to the results from H&E and IHC staining, we found that the FI% and G/T were 33% and 36% higher evaluated using GAGE-7, respectively (both $P < 0.0001$; **Supplementary Figure 2**).

Figure 5 shows the results of the expression of the molecular markers in the patients with and without Ad spermatogonia in the testes (see detailed information in **Supplementary Table 2**). D2-40, OCT3/4, LIN28, and C-KIT showed high frequency in the testes with Ad spermatogonia, whereas the frequency of immunoreactions for PLAP and GAGE-7 was slightly reduced in the patients without Ad spermatogonia (**Figure 5a**). The proportion of cryptorchid testes with and without Ad spermatogonia in different age groups is shown in **Figure 5b**. The results demonstrated that the Ad spermatogonia population disappeared after the age of 2 years. A Freeman–Halton extension of Fisher's exact (2 × 3 contingency table test) was performed to compare the years from occurrence of Ad spermatogonia. The results revealed a significant difference in terms of the number of Ad spermatogonia according to age (1 year, 2 years, and 3 years) ($P = 0.00005$).

DISCUSSION

The study investigated germ cell development in the testes of infant boys with cryptorchidism by staining with new and established germ cell markers, including D2-40, C-KIT, OCT3/4, PLAP, LIN28, and GAGE-7. The results revealed heterogeneity in the expression of these germ cell markers according to the developmental stage in testes from boys with cryptorchidism in relation to age.

We compared the results of the present study to those of our previous study on germ cell development in a healthy population and found that the maturation of germ cells was slightly delayed. However, the pattern of staining of molecular markers over time was the same as that observed in healthy testicular tissue (**Supplementary Table 3**). The present data provide insight into the testicular status in cryptorchid boys. Staining of a combination of the molecular markers studied herein may result in improved understanding of germ cell maturation.

Cryptorchidism is the most common cause of non-obstructive azoospermia.[28] Gonocyte transformation and differentiation into Ad spermatogonia are considered a crucial step in the establishment of an adequate adult germline stem cell pool for future fertility.[7] The aforementioned germ cell development takes place before or around the age of 6 months in normal testicular development.[5,29,30] In cryptorchidism, gonocytes display delayed transformation.[8,31] In previous research, differential gene expression profiling of gonocyte and spermatogonia indicated that DMRTC2, PAX7, T-box transcription factor Brachyury, and telomerase reverse transcriptase played an important role in the development of Ad spermatogonia.[15] In an undescended testis, increases of testosterone and LH reconstitute self-renewal properties of the Ad spermatogonia and induce the expression of retinoic acid responsive genes, such as *NRG1*, *NRG3* and *PAX7*.[15] Although our previous study did not include data on Ad

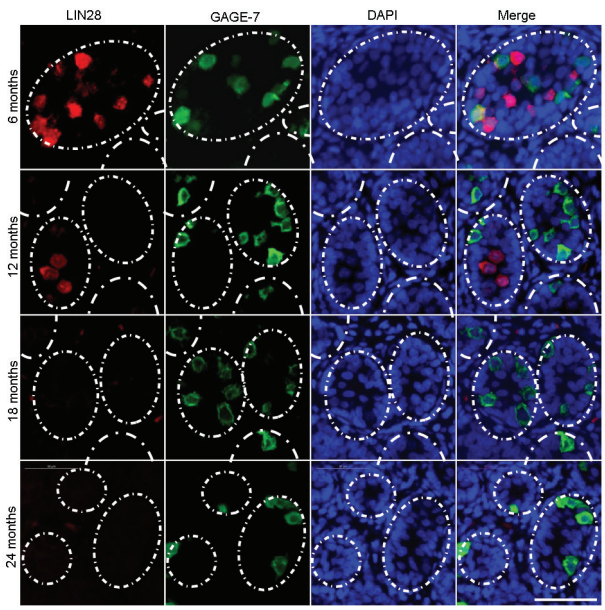

Figure 2: Dual immunofluorescence staining of LIN28 and GAGE-7 in undescended testis aged 6, 12, 18, and 24 months, respectively. Dash lines indicate the seminiferous tubules. Scale bars = 50 μm. LIN28: LIN-28 homolog A; GAGE-7: G antigen 7; DAPI: 4',6-diamidino-2-phenylindole.

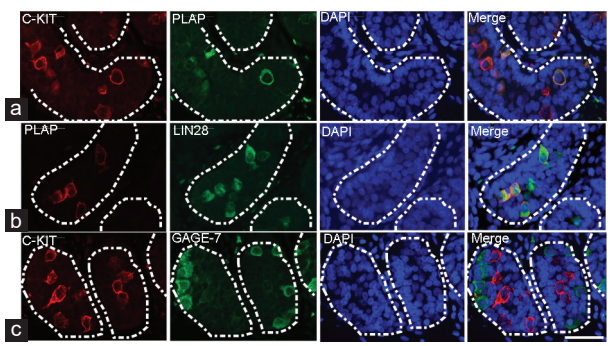

Figure 3: Dual immunofluorescence staining of selected germ cell makers from undescended testes at the age of 10 months. Costaining of (**a**) C-KIT and PLAP, (**b**) PLAP and LIN28 antigens, (**c**) C-KIT and GAGE-7. Dash lines indicate the seminiferous tubules. Scale bars = 50 μm. C-KIT: KIT proto-oncogene; PLAP: placental alkaline phosphatase; LIN28: LIN-28 homolog A; GAGE-7: G antigen 7.

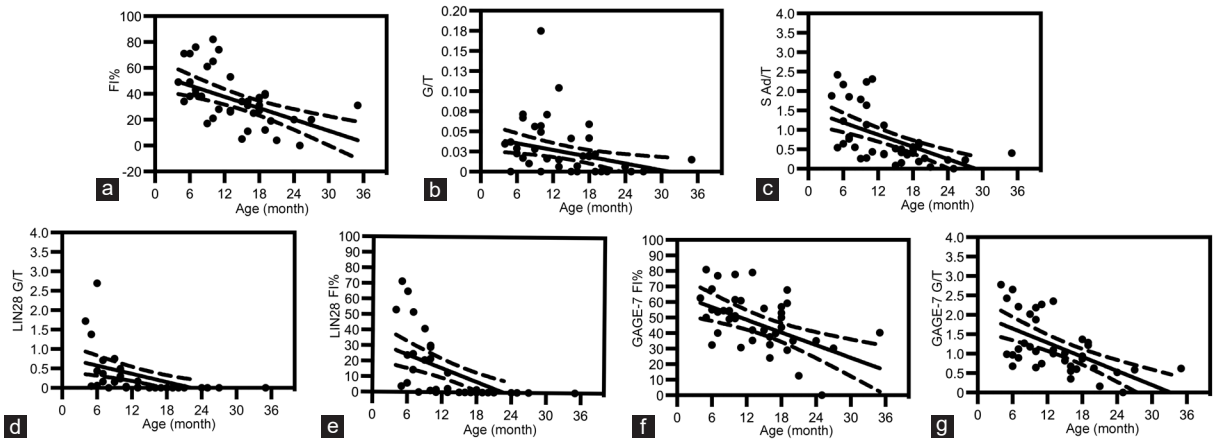

Figure 4: (**a**) Percentage of tubular transverse sections containing germ cells, FI%; (**b**) the number of germ cells per tubular transverse section, G/T; (**c**) the number of spermatogonial stem cells per tubular transverse section, S Ad/T; (**d**) the number of LIN28 positive germ cells per tubular transverse section, G/T; (**e**) percentage of tubular transverse sections containing LIN28 positive germ cells, FI%; (**f**) percentage of tubular transverse sections containing GAGE-7 positive germ cells, FI%; (**g**) the number of GAGE-7 positive germ cells per tubular transverse section, G/T. In each figure, the lines represent a central liner regression line and error bars. FI%: fertility index; G/T: number of germ cells per tubular transverse section; S Ad/T: number of type-A dark spermatogonia per tubular transverse section; LIN28: LIN-28 homolog A; GAGE-7: G antigen 7.

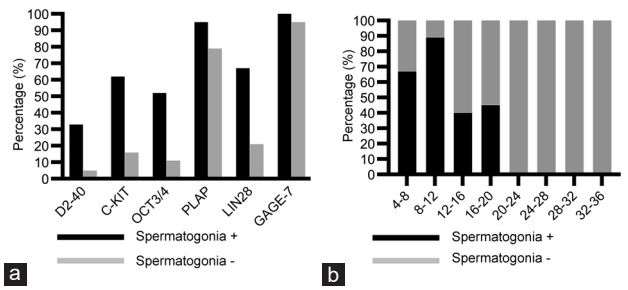

Figure 5: Comparison of the different markers and the population proportions between patients with and without Ad spermatogonia. (**a**) Percentage of boys with positive reaction for each of the immunohistochemical marker subdivided by patients with or without Ad spermatogonia. (**b**) Percentage of boys with and without Ad spermatogonia in relation to age (months). Ad: type A dark.

spermatogonia in healthy testicular tissue, the immunohistochemical staining in the present study confirmed impairment of germ cell number and maturation delay in boys with cryptorchidism (**Supplementary Table 3** and **Supplementary Figure 3**).

A further aspect is to evaluate whether any specific marker or combination of markers will predict the fertility potential. For those cryptorchid boys who have a risk of infertility, cryopreservation of a testicular biopsy containing germ cells is now becoming an fertility preservation option.[32] This highlights the value of the present study, presenting additional data on germ cell number, morphology, and molecular profile of germ cells.[16]

Among cryptorchid boys with a risk of hypospermatogenesis, fertility preservation by freezing a testicular biopsy containing germ cells at an early age, preferentially in connection with surgery for cryptorchidism, is now becoming an option.[32] Thus, the present study adds to the literature by presenting additional data on germ cell numbers, morphologies, and molecular profiles in boys with cryptorchidism and may potentially predict fertility potential.[16]

The D2-40 antibody recognizes the M2A antigen, a marker of adult testicular cancer.[33] Immunoreactivity to D2-40 declines during the movement of gonocytes toward the basement membrane. In the present study, D2-40 disappeared firstly in germ cells in the biopsy material, whereas its expression persisted in Sertoli cells. The D2-40-positive cells likely represented gonocytes moving toward the basement membrane. Interestingly, OCT3/4 positive germ cell expression was found throughout the development period in boys till aged 16 months but mainly in a central location. In newborn normal mice, Li *et al.*[34] demonstrated that all gonocytes were Oct3/4-positive on postnatal day 0 and day 2, whereas very few germ cells were OCT3/4-negative on day 4. The number of OCT3/4-positive germ cells per tubule started to decrease, whereas those of OCT3/4-negative germ cells significantly increased from day 6 to day 10. The number of OCT3/4-positive germ cells per tubule was similar on days 6, 8, and 10, and the majority of these germ cells were located in the basement membrane. However, the number of OCT3/4-negative germ cells per tubule increased significantly from day 6 to day 10. Although results from rodent studies may differ from those of human studies, it seems reasonable that positive gonocyte staining for D2-40 and OCT3/4 is associated with phases of migration to the basement membrane. LIN28, an RNA-binding pluripotent stem cell factor, was detected in both centrally and peripherally located germ cells in our study and detected throughout the germ cell developmental period longer than OCT3/4-positive cells. Overall, our results generally confirm the findings of Aeckerle *et al.*[19] who proposed that LIN28-positive cells were spermatogonial stem cells. In the present study, at a very young age, LIN28 and GAGE-7 were colocalized, but almost no costaining was observed after the age of 6 months (**Figure 2**). To our knowledge, this is the first study to investigate germ cell development using GAGE-7 staining in infant testes and to confirm GAGE-7 expression in germ cells in biopsies from undescended testes up to the age of 35 months.

Our study demonstrated variability in specific germ cells markers in relation to age and position within the seminiferous tubules of testes from cryptorchid boys. Migration from the center to the periphery in testicular tubules is important for the transformation of gonocytes into spermatogonia. Based on the present data, we hypothesize that the expression pattern observed reflected this maturation process. According to the presence of different markers (**Table 1**), we hypothesize that D2-40, C-KIT, OCT3/4, and LIN28 in germ cell populations, including gonocytes and spermatogonia, either alone or simultaneously with other markers, occur in the early stages of development. Germ cells in a peripheral location in the seminiferous cords near the basement membrane may represent spermatogonial stem cells. In later stages of

germ cell development, PLAP and GAGE-7 are probably expressed in Ad and type A pale (Ap) spermatogonia cells located in the basement membrane. In the present study, the frequencies of D2-40, OCT3/4, C-KIT, and LIN28 expression were higher in the biopsy materials with Ad spermatogonia as compared with those without Ad spermatogonia. PLAP and GAGE-7 expressions were slightly reduced in the patients without Ad spermatogonia. Hadziselimovic *et al.*[35,36] found that *LIN28B*, *GAGE1*, *GAGE3*, and *GAGE12B* expression were downregulated in a high infertility risk group of cryptorchid boys with impaired mini-puberty. To our knowledge, none of the markers visualized all germ cells. Therefore, co-immunostaining with other markers, as was done in the present study, may be necessary for a more detailed understanding of germ cell development. The expression patterns of the CD99, D2-40, OCT3/4, C-KIT, and PLAP markers in the present study were consistent with those reported in previous studies.[12,13,37] In cryptorchid boys, germ cells positive for OCT3/4 and C-KIT were found throughout the 1st year of life.[12,13] In our study, OCT3/4-positive germ cells were detected in cryptorchid boys at the age of 13 months in accordance with the findings of some previous studies on undescended testes.[12,13] The latter may indicate delayed maturation. In the present study, C-KIT was not expressed after the age of 16 months. In contrast, in infants without cryptorchidism, we previously showed that C-KIT was expressed in 38% of 24-month-old infants. This finding is likely explained by the modest patient sample in the present study. Only 13 patients were aged between 16 and 24 months, and none were C-KIT positive. However, a previous study on immunohistochemical staining of cryptorchid testes in boys aged 12–24 months reported that 33% (108/324) of the testes were C-KIT positive.[13] Other research reported that the percentage of PLAP-positive germ cells decreased in early infancy in biopsies of undescended testes.[38] In contrast, PLAP-positive germ cells were present in all specimens in the present study, irrespective of age, which is in accordance with our observations of 1521 consecutive testicular biopsies from cryptorchid boys aged from 1 month to 16.5 years.[13] Collectively, the data supports the notion of a maturation delay in the testes of boys with cryptorchidism. This delay may be due to defective mini-puberty or a high temperature in the environment of an undescended testis.

We calculated the FI% and G/T based on the results of IHC and IF staining. To determine the G/T and Ad/T, we examined at least 100 cross sections of the seminiferous tubules. As Ad spermatogonia are relatively rare, it is important that they are counted in at least 100 cross sections. Fifty cross sections were included in the IF evaluation because most of the IF-stained sections were unable to reach 100 cross sections. However, in several cases, we counted the cells in at least 100 cross sections also in the IF staining. Moreover, according to previous research, 25 cross-sectioned tubules are sufficient.[27]

For simplicity, we performed a regression analysis merely to illustrate the trend in decreasing G/T according to age. However, as the germ cell number (G/T) physiologically decreases in patients from the age of 1–3 years, the regression analysis as presented has no pathophysiological importance. The number of germ cells counted with GAGE-7 staining was surprisingly higher than the number counted by conventional staining. This may reflect enhanced sensitivity of GAGE-7 staining in the identification of germ cells. In addition, co-immunostaining of LIN28 and GAGE-7, LIN28 and PLAP, PLAP and C-KIT, and CKIT and GAGE-7 uncovered the complexity of germ cell populations during testicular development. In the future, triple marker co-immunostaining or single-cell sequencing of infantile male germ cells over a specific time span can be performed to uncover germ cell developmental kinetics in the early stages after birth.

There are limitations to our study. Due to ethical considerations, this study did not include biopsy specimens from boys without cryptorchidism. However, normal values for D2-40, C-KIT, OCT3/4, and PLAP were based on those found in forensic material analyzed in our laboratory.[26] In addition, the G/T number was markedly reduced in cryptorchidism cases after 1 year (**Supplementary Figure 3**). Likewise, the proportion of cryptorchidism patients with Ad spermatogonia significantly reduced after the 1st year of life, suggesting that Ad spermatogonia may not survive after the age of 2 years (**Figure 5b**). Our data support the suggestion that early orchiopexy reduces the risk of infertility. Future studies should include more samples, preferably in combination with gene expression studies to complement the present observations. Furthermore, whether GAGE-7 is expressed only in germ cells needs to be verified. The latter could be accomplished using fluorescence-activated cell sorting purified cell populations or isolated human spermatogonial stem cells from cultures.

CONCLUSION

Antibodies for specific germ cell markers were able to distinguish different types of germ cells during testicular development in cryptorchid boys. D2-40, LIN28, and C-KIT showed high expression rates in the testes containing Ad spermatogonia. The germ cell number per tubular cross sections counted by IF staining of GAGE-7 was significantly higher than the values obtained by conventional methods. Co-immunostaining of germ cell markers revealed heterogeneity of germ cell development in testicular biopsies from boys with cryptorchidism.

AUTHOR CONTRIBUTIONS

LHD carried out conception and design of the study, data acquisition and analysis, and drafted the manuscript. SH carried out data acquisition and analysis and drafted the manuscript. ECL, KK, and DC carried out data acquisition and analysis. JT and CYA carried out conception and design of the study and data analysis. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Distribution of different molecular positive germ cells in testes of cryptorchid boys aged 4 to 35 months

Distribution	D2-40 % (positive No./total positive No.)	C-KIT % (positive No./total positive No.)	OCT3/4 % (positive No./total positive No.)	PLAP % (positive No./total positive No.)	LIN28 % (positive No./total positive No.)	GAGE-7 % (positive No./total positive No.)	*_P_-value
Only central	0	19 (3/16)	47 (7/15)	0	0	0	<0.0001
Mainly central	11 (1/9)	69 (11/16)	53 (8/15)	0	26 (5/19)	0	<0.0001
Equally central and peripheral	89 (8/9)	12 (2/16)	0	32 (12/38)	74 (14/19)	3 (1/39)	<0.0001
Mainly peripheral	0	0	0	68 (26/38)	0	97 (38/39)	<0.0001
Only peripheral	0	0	0	0	0	0	-

*Chi square test.

Supplementary Table 2: Percentage of boys with positive reaction for each of the immunohistochemical marker subdivided by patients with or without Ad spermatogonia according to age.

Age (months)	Ad spermatogonia (+ or -, all)	n (boys)	D2-40 (%, No./total No.)	C-KIT (%, No./total No.)	OCT3/4 (%, No./total No.)	PLAP (%, No./total No.)	LIN28 (%, No./total No.)	GAGE (%, No./total No.)	*_P_-value
4 - < 8	+	6	83 (5/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	100 (6/6)	0.3987
	-	3	33 (1/3)	67 (2/3)	67 (2/3)	100 (3/3)	100 (3/3)	100 (3/3)	0.2667
	all	*9*	*67 (6/9)*	*100 (9/9)*	*89 (8/9)*	*100 (9/9)*	*100 (9/9)*	*100 (9/9)*	*0.0365*
8 - < 12	+	8	25 (2/8)	75 (6/8)	63 (5/8)	100 (8/8)	75 (6/8)	100 (8/8)	0.0077
	-	1	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	100 (1/1)	0.3062
	all	*9*	*22 (2/9)*	*56 (5/9)*	*56 (5/9)*	*100 (9/9)*	*78 (7/9)*	*100 (9/9)*	*0.0019*
12 - < 16	+	2	0 (0/2)	50 (1/2)	0 (0/2)	100 (2/2)	50 (1/2)	100 (2/2)	0.1562
	-	3	0 (0/3)	33 (1/3)	0 (0/3)	100 (3/3)	33 (1/3)	100 (3/3)	0.0274
	all	*5*	*0 (0/5)*	*40 (2/5)*	*0 (0/5)*	*100 (5/5)*	*40 (2/5)*	*100 (5/5)*	0.0011
16 - < 20	+	5	0 (0/5)	0 (0/5)	0 (0/5)	80 (4/5)	20 (1/5)	100 (5/5)	0.0004
	-	6	0 (0/6)	0 (0/6)	0 (0/6)	67 (4/6)	0 (0/6)	100 (6/6)	<0.0001
	all	*11*	*0 (0/11)*	*0 (0/11)*	*0 (0/11)*	*73 (8/11)*	*9 (1/11)*	*100 (11/11)*	*<0.0001*
20 - < 24	+	0	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	-
	-	2	0 (0/2)	0 (0/2)	0 (0/2)	50 (1/2)	0 (0/2)	100 (2/2)	0.095
	all	*2*	*0 (0/2)*	*0 (0/2)*	*0 (0/2)*	*50 (1/2)*	*0 (0/2)*	*100 (2/2)*	*0.095*
24 - < 28	+	0	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	-
	-	3	0 (0/3)	0 (0/3)	0 (0/3)	67 (2/3)	0 (0/3)	67 (2/3)	0.068
	all	*3*	*0 (0/3)*	*0 (0/3)*	*0 (0/3)*	*67 (2/3)*	*0 (0/3)*	*67 (2/3)*	*0.068*
32 - < 36	+	0	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	0 (0/0)	-
	-	1	0 (0/1)	0 (0/1)	0 (0/1)	100 (1/1)	0 (0/1)	100 (1/1)	0.3062
	all	*1*	*0 (0/1)*	*0 (0/1)*	*0 (0/1)*	*100 (1/1)*	*0 (0/1)*	*100 (1/1)*	*0.3062*
In total	+	21	33 (7/21)	62 (13/21)	52 (11/21)	95 (20/21)	67 (14/21)	100 (21/21)	<0.0001
	-	19	5 (1/19)	16 (3/19)	11 (2/19)	79 (15/19)	21 (4/19)	95 (18/19)	<0.0001
	all	*40*	*20 (8/40)*	*40 (16/40)*	*33 (13/40)*	*88 (35/40)*	*48 (19/40)*	*98 (39/40)*	<0.0001

*Chi square test

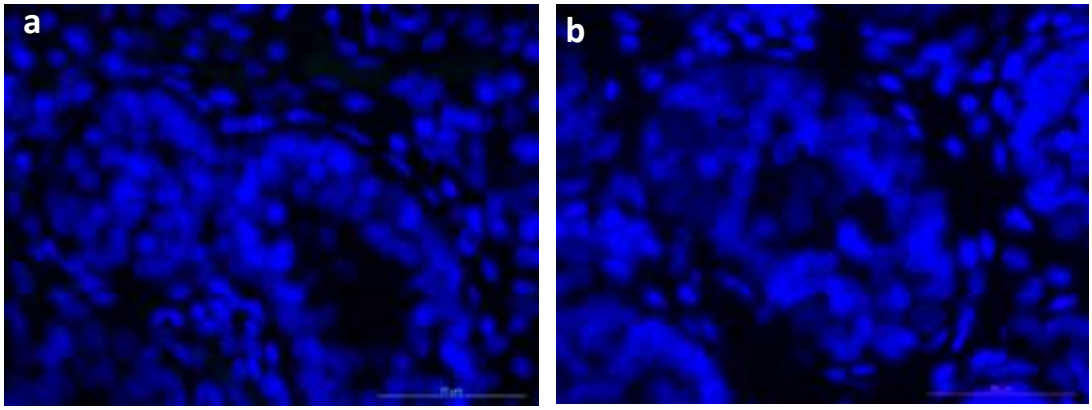
Supplementary Table 3: Percentage of boys with a positive reaction to each of the immunohistochemical markers grouped by age in years.

Group 1 from normal boys. Forensic Medicine Material (Reference 22 in main text)

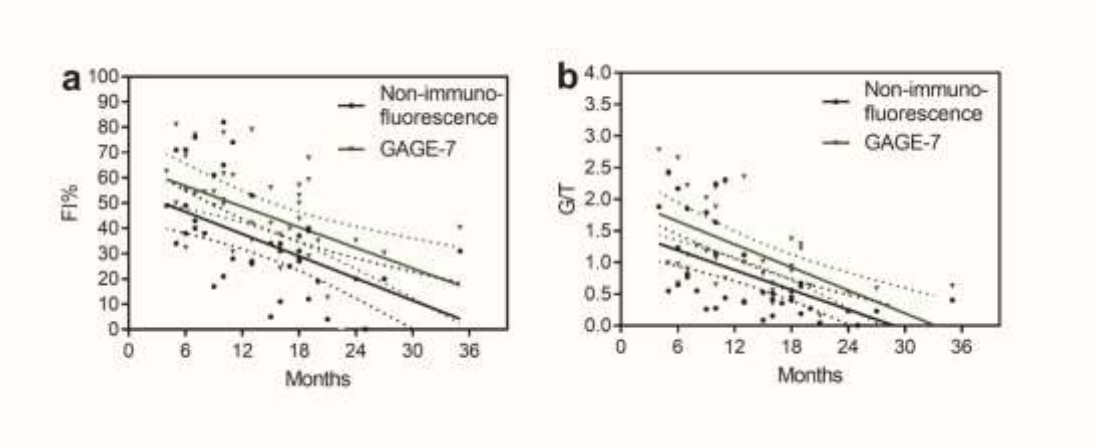
Age	n (boys)	D2-40 pos (%)	C-Kit pos (%)	OCT3/4 pos (%)	PLAP pos (%)
0-< 1/2 years	32	57%	86%	73%	100%
1/2 -< 1 years	10	0%	70%	10%	100%
1-<2years	17	0%	47%	0%	100%

Group 2 from boys with cryptorchidism. Present materials

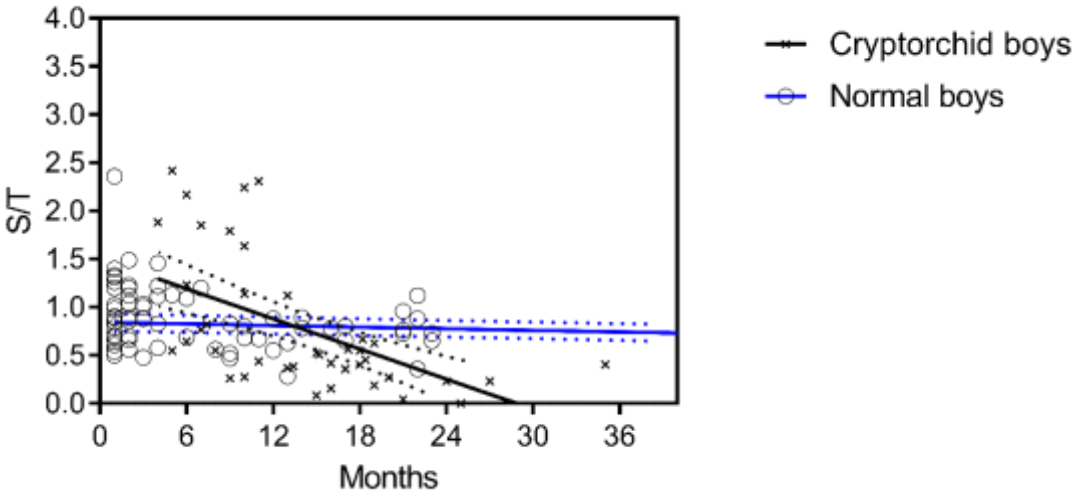
Age	n (boys)	D2-40 pos (%)	C-kit pos (%)	OCT3/4 pos (%)	PLAP pos (%)
0-< 1/2 years	3	67%	67%	80%	100%
1/2 -< 1 years	15	40%	80%	70%	100%
1-<2years	18	0%	11%	0%	78%



Supplementary Figure 1: Negative controls for immunofluorescence. **a**: Merged for the DAPI and FITC-labelled donkey anti-mouse IgG. **b**: Merged for DAPI and Alexa Fluor® 594 labelled donkey anti-rabbit IgG. Scale bars: 50 µm.



Supplementary Figure 2: Comparison of fertility index FI% and G/T value generated from non-immunofluorescence (non-IF) and GAGE-7 immunofluorescent studies. In every figure, the lines represent a liner regression line.



Supplementary Figure 3: Number of germ cells including Ad spermatogonia per transverse tubule according to normal boys (linear regression, *P*=0.003) and cryptorchid boys (linear regression, *P*=<0.0001).