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Glycogen Synthase Kinase-3 Isoform Variants and Their Inhibitory Phosphorylation in Human Testes and Spermatozoa

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Purpose: To clarify (phospho-) glycogen synthase kinase-3 (GSK3) isoform variants in the germline and soma of human testes and spermatozoa.

Materials and Methods: GSK3 isoform variants in normospermatogenic and Sertoli cell-only (SCO) testicular biopsies and spermatozoa were examined.

Results: In normospermatogenic testes, GSK3α and GSK3β variants 1 and 2 different in low complexity region (LCR) were expressed and their levels were decreased in SCO testes. *GSK3β* variant 3 was only expressed in SCO testes. GSK3β as well as GSK3α, the dominant isoforms in testes were decreased in SCO testes. In normospermatogenic testes, GSK3β were found in spermatogonia and markedly decreased in meiotic germ cells in which GSK3α was dominant. p-GSK3α/β were marginal in spermatogonia and early spermatocytes. In SCO testes, GSK3α/β immunoreactivity in seminiferous epithelia was weaker than those of normospermatogenic testes whereas p-GSK3α/β(Ser) immunoreactivity was visibly increased in Sertoli cells. GSK3α was dominant in ejaculated spermatozoa in which GSK3α and p-GSK3α(Ser) were found in the head, midpiece, and tail. In acrosome-reacted spermatozoa, GSK3α was found in the equatorial region of head, midpiece, and tail, and p-GSK3α(Ser) was only found in midpiece. During sperm capacitation, p-GSK3α(Ser) was significantly increased together with phosphotyrosine proteins and motility.

Conclusions: In human male germ cells, GSK3 isoforms different in LCRs switch from GSK3β to GSK3α during meiotic entry, suggesting the isoform-specific roles of GSK3α and GSK3β in meiosis and stemness or proliferation of spermatogonia, respectively. In dormant Sertoli cells of SCO testes kinase activity of GSK3 might be downregulated via inhibitory phosphorylation. In spermatozoa, inhibitory phosphorylation of GSK3α might be coupled with activation of motility during capacitation.

Keywords: Glycogen synthase kinase 3; Human; Sertoli cell-only; Spermatozoa; Testis

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INTRODUCTION

Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase that is the central gatekeeper involved in various cellular processes including cell lineage specification, glucose metabolism, and apoptosis [1-3]. GSK3 is conserved in all eukaryotes and exists in α and β isoforms different in low complexity regions (LCRs) in mammals. LCRs are amino acid sequences that contain repeats of single amino acids or short amino acid motifs and associated with protein structure, genetic recombination, and protein-protein interactions [4,5]. In yeast, Rim11p (regulator of inducer of meiosis), a key member of the nutritional cascade governing meiosis, is a homolog of GSK3β [6]. In mammals, GSK3 is a key regulator of proliferation and pluripotency in male germline stem cells [7,8], suggesting that GSK3 is an essential key regulator in eukaryotic gametogenesis. In mice, the gene deletion of GSK3α decreased sperm motility, resulting in infertility [9]. In mammalian spermatozoa, the inhibition of GSK3α *via* phosphorylation on Ser residue is positively associated with sperm motility [10,11]. In humans, valproic acid, a GSK3 inhibitor disrupted spermatogenesis and sperm motility, suggesting the involvement of GSK3 in male fertility [12,13]. Nonetheless, the functional expression of the GSK3 isoform variants remains unsolved in human testes. In this study, GSK3 isoform variants and their phosphorylation were investigated in the normospermatogenic and Sertoli cell-only (SCO) syndrome human testes and spermatozoa.

MATERIALS AND METHODS

1. Ethics statement

Experiments with human samples were performed in accordance with the Institutional Review Board (IRB) of Cheil General Hospital (approval number: CGH-IRB-2014-29). For the biochemical analyses in this study, informed consent was obtained from every patient.

2. Testicular biopsy and sperm collection

This study included male patients who visited Cheil General Hospital between January 1, 2015 and November 31, 2018, and received care from a single physician. Totally six testicular biopsies were classified as normospermatogenesis (n=3) and SCO (n=3) according to the cellular composition of the seminiferous tubules and subjected to further analysis. Semen samples were obtained from 4 healthy men by masturbation after 3 to 5 days of sexual abstinence and subjected to sperm isolation after liquefaction within 30 minutes.

3. *In silico* analysis of the protein domains of the GSK3 variants

Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) was used to determine the domain structures of the GSK3α and GSK3β variants. Briefly, human GSK3α (NP_063937.2), GSK3β isoform 1 (NP_002084.2), GSK3β isoform 2 (NP_001139628.1), and GSK3β isoform 3 (NP_001341525.1) protein sequences were analyzed by SMART. Catalytic domain and LCRs were annotated in sequence.

4. RNA preparation and RT-PCR

The transcript variants of *GSK3α* and *GSK3β* in human testes were determined using specific primers for *GSK3α*, *GSK3β1*, *β2*, and *β3* mRNA (Fig. 1). Total RNA from the testis biopsies was isolated using QIAzol Lysis Reagent (79306; QIAGEN, Valencia, CA, USA), precipitated with isopropanol, dissolved in diethyl pyrocarbonate (Sigma-Aldrich Korea, Seoul, Korea) treated water, and quantified. Following the purity checking RNA was reverse transcribed with ReverTra Ace™ qPCR RT Master Mix (Toyobo, Osaka, Japan). PCR was performed with a PCR Master Mix (RR310A; TaKaRa, Kusatsu, Japan). *RPL7* mRNA was used as an internal control. The primer sequences and amplicon sizes for the RT-PCR are listed in Table 1. The PCR products were separated on 2% agarose gels containing 20,000× RedSafe (21141; iNtRON Biotechnology, Seongnam, Korea) and were photographed under UV light.

5. Incubation of spermatozoa

Human spermatozoa were isolated from freshly ejaculated semen from healthy men. Tyrode's basal medium (TBM, absent of Ca^{2+}, bovine serum albumin [BSA], and bicarbonate) was overlaid on the ejaculated semen, and spermatozoa were allowed to swim up for 20 minutes in a CO_2 incubator at 37°C. Motile spermatozoa were harvested, washed in TBM, and centrifuged at 500×*g* for 5 minutes. After a second wash and centrifugation, the sperm concentration was adjusted to 1×10^6 cells/m in modified Tyrode's complete medium (TCM, containing Ca^{2+}, BSA, and bicarbonate) and incubated

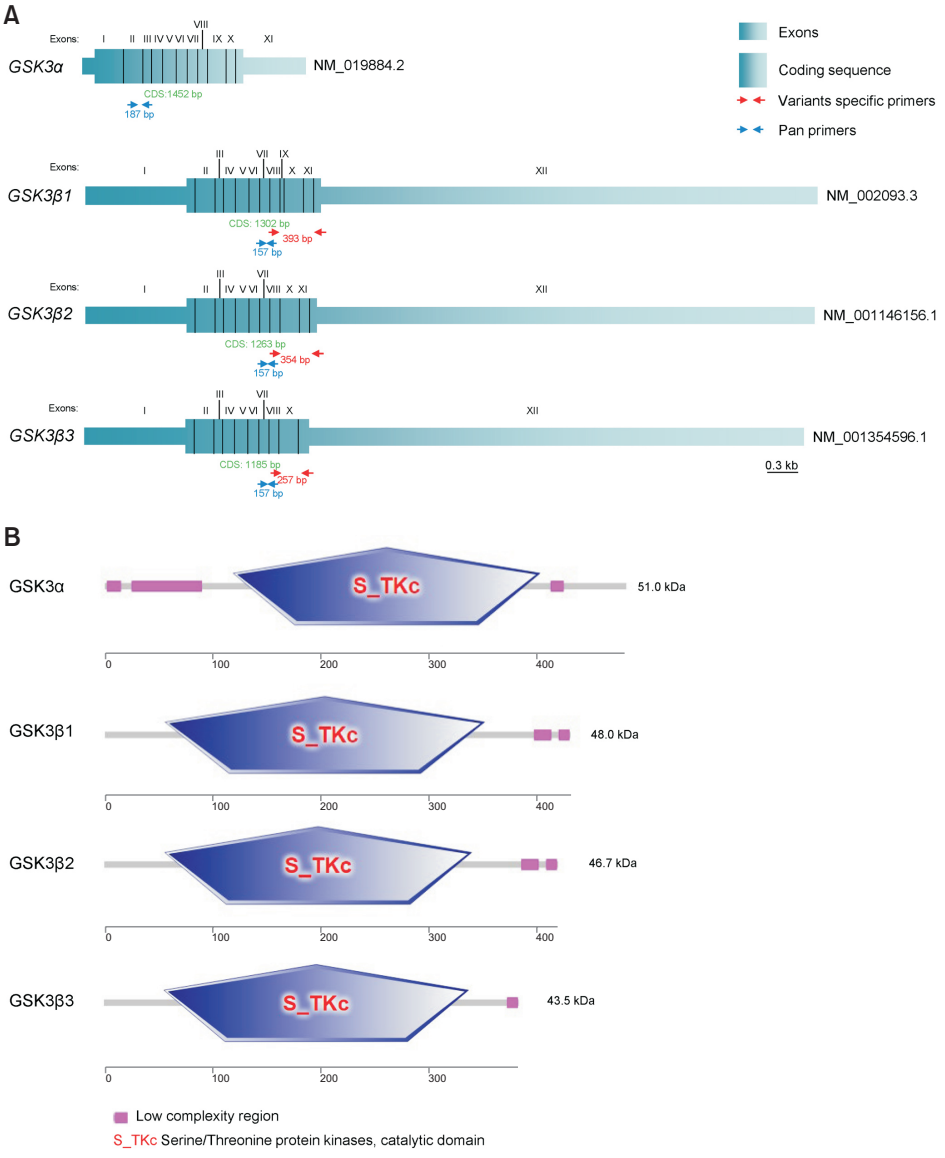

Fig. 1. Human GSK3 isoforms. (A) Transcript variants of GSK3 isoforms. RT-PCR primer locations of the *GSK3α* and *GSK3β* transcript variants. Detailed primer sequences are shown in Table 1. (B) Protein domains of each transcript variant.

Table 1. Primer sequences for RT-PCR

Gene		Primer sequences	Amplicon (bp)	GenBank Acc. No.
GSK3α	F	5'-CTGGTGCTGGAATATGTGCC-3'	195	NM_019884.2
	R	5'-AGCAGTGTCAGGGTCCACC-3'		
GSK3β (pan)	F	5'-AGGCAATTGCACTGTGTAGC-3'	157	NM_002093.3
	R	5'-GAAGTTGAAGAGTGCAGGTGTG-3'		NM_001146156.1
				NM_001354596.1
GSK3β (1, 2, 3)	F	5'-CCCTCAAATTAAGGCACATCC-3'	β1: 393	NM_002093.3
	R	5'-GCATTATTGGTCTGTCCACGG-3'	β2: 354	NM_001146156.1
			β3: 257	NM_001354596.1
RPL7	F	5'-TCAATGGAGTGAGCCCAAAG-3'	246	NM_000971.3
	R	5'-CAAGAGATCGAGCAATCAAAG-3'		

for 3 hours in a CO_2 incubator at 37°C. After incubation, spermatozoa were subjected to protein extraction and immunocytochemistry.

6. Computer-assisted sperm analysis (CASA)

Sperm motility analysis was conducted using video recording and CASA. Briefly, 20-μL sperm samples in

media were dispensed onto a pre-warmed MAKLER® counting chamber (Irvine Scientific, Santa Ana, CA, USA) at 37℃. Movies were recorded on a Nikon Diaphot microscope equipped with a CoolSnap EZ CCD camera (Photometrics, Tucson, AZ, USA) controlled through iSPERM software (CNC Biotech, Suwon, Korea).

7. Western blot analysis

Tissues and spermatozoa were homogenized in phosphate buffered saline (PBS) containing 1% Triton-X-100 and 1% (v/v) protease and phosphatase inhibitor cocktail. After sonication 5 times for 5 seconds at 4℃. lysates were centrifuged at 14,000 rpm for 20 minutes, and the supernatants were resolved by SDS-PAGE, After transfer to nitrocellulose membrane western blotting was performed with antibodies specific for GSK3α/β (#5676; Cell Signaling, Beverly, MA, USA), p-GSK3α/β(Ser21/9) (#9327; Cell Signaling), phosphotyrosine (p-Tyr; 05-321; Millipore, Temecula, CA, USA), β-tubulin (ab108342; Abcam, Cambridge, UK), and GAPDH (sc-25778; Santa Cruz, Dallas, TX, USA). The primary antibodies used in this study are listed in Table 2. The antibodies were diluted 1:2,000–5,000 in 5% skim milk in Tris-buffered saline (TBS) and incubated with the membranes overnight at 4℃. After rinsing three times with TBS/0.1% Tween 20 (TBST), the membranes were incubated with peroxidase-labeled goat anti-rabbit IgG (ab6721; Abcam) diluted 1:5,000 in 5% skim milk in TBST for 1 hour. After washing three times with TBST, signals were detected with Fusion SL (Vilber Lourmat; Marne-la-Vallée, France) and ECL Prime Western Blotting Detection Reagent (RPN2232; Amersham Bioscience, Amersham, UK) according to the manufacturers' instructions. As an internal control, GAPDH and β-tubulin were probed in testes and sperm samples, respectively. The band intensities were analyzed in four sperm samples using the ImageJ Ver.1.51j8 (National Institutes of Health, Bethesda, MD, USA).

8. Immunohistochemistry and of GSK3 in human testis biopsies

Upon biopsy, testis tissues were fixed in Bouin's solution (Sigma-Aldrich Korea) for 16 hours. After dehydration and clearing, the testis tissues were embedded in Paraplast (Sigma-Aldrich Korea), and 5-μm-thick sections were mounted on poly-L-lysine-coated slides and subjected hematoxylin and eosin (H&E) staining and immunohistochemistry. After deparaffination and rehydration, the slides were blocked in 5% goat serum in PBS. Subsequently, the slides were incubated in a humidified chamber overnight at 4℃ with antibodies specific for GSK3α (#4337; Cell Signaling), GSK3β (#12456; Cell Signaling), p-GSK3α(Ser21) (sc-101690; Santa Cruz), and p-GSK3β(Ser9) (sc-11757-R; Santa Cruz) diluted 1:1,000 in 1.5% goat serum in PBS. After three times washes in PBS, the slides were incubated for 1 hour in goat anti-rabbit IgG H&L (ab6721; Abcam) diluted 1:200 in 1.5% goat serum. After washed three times in PBS, a coloring reaction was performed with ImmPACT DAB substrate (SK-4105; Vector Labs, Burlingame, CA, USA). The nuclei were stained with Harris hematoxylin and permanently mounted with Canada balsam.

9. Immunocytochemistry of GSK3 in human spermatozoa

Spermatozoa were smeared on poly-L-lysine-coated slides, fixed in acetone:methanol (1:1) at 4℃ for 10

Table 2. List of antibodies

Antibody	Catalog number	Host	Company
GSK3α/β	#5676	Rabbit	Cell Signaling, Beverly, MA, USA
p-GSK3α/β(Ser21/9)	#9327	Rabbit	Cell Signaling
GSK3α	#4337	Rabbit	Cell Signaling
GSK3β	#12456	Rabbit	Cell Signaling
p-GSK3α(Ser21)	sc-101690	Rabbit	Santa Cruz, Dallas, TX, USA
p-GSK3β(Ser9)	sc-11757-R	Rabbit	Santa Cruz
Phosphotyrosine	05-321	Mouse	Millipore, Temecula, CA, USA
β-tubulin	ab108342	Rabbit	Abcam, Cambridge, UK
GAPDH	sc-25778	Rabbit	Santa Cruz

minutes, and air dried. The slides were blocked in 5% donkey serum in PBS and incubated with GSK3α/β and p-GSK3α(Ser21) (sc-101690; Santa Cruz) antibody diluted in 1.5% donkey serum in PBS in a humidified chamber overnight at 4℃. Normal rabbit IgG (ab27478; Abcam) was used as a negative control to replace the primary antibodies. After washing three times in PBS, signal was developed with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (ab150061; Abcam) diluted in 1.5% donkey serum in PBS for 1 hour at room temperature. For acrosome visualization, the slides were incubated with rhodamine-labeled peanut agglutinin (PNA; RL-1072; Vector Labs) diluted in 1.5% donkey serum in PBS for 1 hour at room temperature. After washing three times in PBS, nuclear staining and mounting were conducted with ProLong™ Gold Antifade mounting medium containing DAPI (P36931; Invitrogen, Carlsbad, CA, USA). Images were captured with a fluorescence microscope equipped with a cooled CCD (DP71; Olympus, Tokyo, Japan). The subcellular fluorescence intensities were analyzed in five sperm images using the ImageJ Ver.1.51j8.

10. Statistical analysis

Statistical analyses were performed by SPSS (version 17.0; SPSS Inc., Chicago, IL, USA) using an unpaired, two-tailed Mann–Whitney U test. Statistical significance was defined as *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

1. GSK3 isoform variants in human testes

Although the coding sequences were similar, the non-coding 5' and 3' regions differed widely among the *GSK3* transcript variants. The amino acid (aa) sequences of the GSK3 isoform variants differed between 395 and 484 aa: GSK3α, 484 aa; GSK3β1, 434 aa; GSK3β2, 421 aa; GSK3β3, 395 aa. The protein domains encoded by each variant consisted of a central serine/threonine protein kinase catalytic domain (S_TKc) of about 304 aa and LCRs in the N- and C- terminals that differed in number, position, and length between GSK3α and GSK3β (Fig. 1).

2. Expression of GSK3 isoform variants in human testes

In the normospermatogenic testes, a single amplicon for *GSK3α* mRNA was detected by RT-PCR, together with *GSK3β1* and *GSK3β2* transcript variants. Although *GSK3α* and *GSK3β* transcript variants were expressed in the SCO testes, the *GSK3α*, *GSK3β* (pan), and *GSK3β2* mRNA levels in the SCO testes were lower than those in the normospermatogenic testes. *GSK3β3* mRNA was detected in SCO testes, but not in normospermatogenic testes. In western blots of the human testis tissues, 46–51 kDa GSK3 antibody immunoreactive bands were detected. The 51-kDa protein corre-

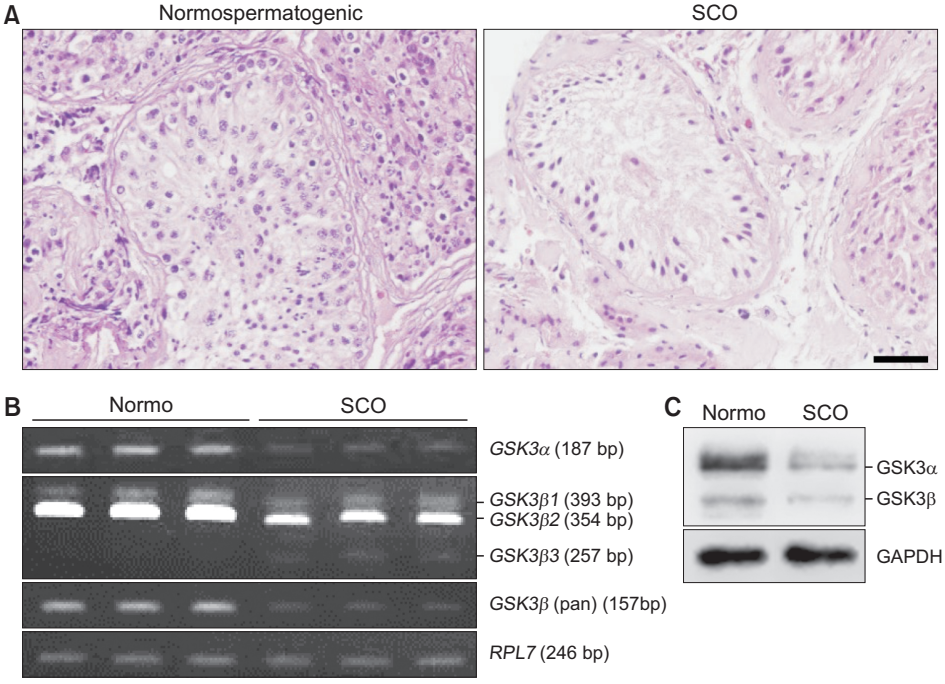

Fig. 2. Expression of GSK3 in human testes. (A) Representative H&E staining of normospermatogenic and Sertoli cell-only (SCO) testes biopsies. Bar=50 μm. (B) RT-PCR of *GSK3α* and *GSK3β* transcript variants in human normospermatogenic (n=3) and SCO (n=3) testes biopsies. *GSK3α* and *β* mRNA levels were reduced in SCO testes, in which *GSK3β3* mRNA was expressed. (C) Western blot analysis of GSK3α/β in human testis biopsies. The GSK3α levels were higher in normospermatogenic testes than those of GSK3β. In SCO testis, GSK3α/β levels were lower than those of the normospermatogenic testes.

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sponding to GSK3α was abundant in the testes. Among the translation products of GSK3β transcript variants, the 46-kDa form corresponding to GSK3β2 was visibly expressed but the 48-kDa form (GSK3β1) and the 44-kDa form (GSK3β3) were not detected. In the SCO testes, the GSK3α and GSK3β levels were lower than those in the normospermatogenic testes (Fig. 2). In immunohistochemical analyses of normospermatogenic testes, GSK3α immunoreactivity was found in the early spermatocytes, elongating/elongated spermatids, and Sertoli cells, but it was weak to marginal in the spermatogonia, peritubular cells, and Leydig cells. In addition, p-GSK3α(Ser21) immunoreactivity was found in Sertoli cells, peritubular cells, and Leydig cells, but it was marginal in spermatogonia, preleptotene, and leptotene spermatocytes. GSK3β immunoreactivity was found in spermatogonia, round spermatids, elongated/elongating spermatids, Sertoli cells, peritubular cells, and Leydig cells, but it was weak to marginal in preleptotene and pachytene spermatocytes. In normospermatogenic testes, p-GSK3β(Ser9) immunoreactivity was primarily found in Sertoli cells and Leydig cells, and it was weak to marginal in peritubular cells, spermatogonia, spermatocytes, and elongating/elongated spermatids. The immunoreactivities of GSK3α/β and p-GSK3α/β(Ser21/9) in testicular cells from normospermatogenic testes are summarized in Table 3. In SCO testes, GSK3α immunoreactivity was found in Sertoli cells, Leydig cells, and peritubular cells, and the p-GSK3α(Ser21) immunoreactivity in Sertoli cells was markedly increased compared to that of the normosperamtogenic testes. The GSK3β immunoreactivity was found in Sertoli cells, peritubular cells, and Leydig cells, and p-GSK3β(Ser9) immunoreactivity in Sertoli cells was markedly increased compared to that of the normospermatogenic testes (Fig. 3).

3. Expression of GSK3 isoforms in human spermatozoa

In the acrosome-intact spermatozoa, GSK3α/β was found in the acrosome and post-acrosomal region, midpiece, and principal piece of the tail. p-GSK3α(Ser21) was primarily found in the post-acrosomal region of the head and midpiece but weak in principal piece of tail. In the acrosome-reacted spermatozoa exhibiting characteristic PNA labeling in the equatorial region of the head, GSK3α/β was found in the equatorial region, midpiece, and principal piece of the tail whereas p-GSK3α(Ser21) was found only in the midpiece. No

Table 3. Summary of GSK3 expression in human testes

GSK3 isoform	Expression levels in normospermatogenesis							
	Spermatogonia	Preleptotene-leptotene spermatocytes	Zygotene-pachytene spermatocytes	Round spermatids	Elongating/elongated spermatids	Sertoli cells	Peritubular cells	Leydig cells
GSK3α	+/-	++	+++	+++	+++	++	+	+/-
p-GSK3α (Ser21)	+/-	+/-	-	-	-	++	++	+++
GSK3β	+++	+/-	+/-	++	+++	++	++	++
p-GSK3β (Ser9)	+/-	+/-	-	-	+/-	++	+	++

-: absent, +/-: marginal, +: weak, ++: moderate, +++: strong.

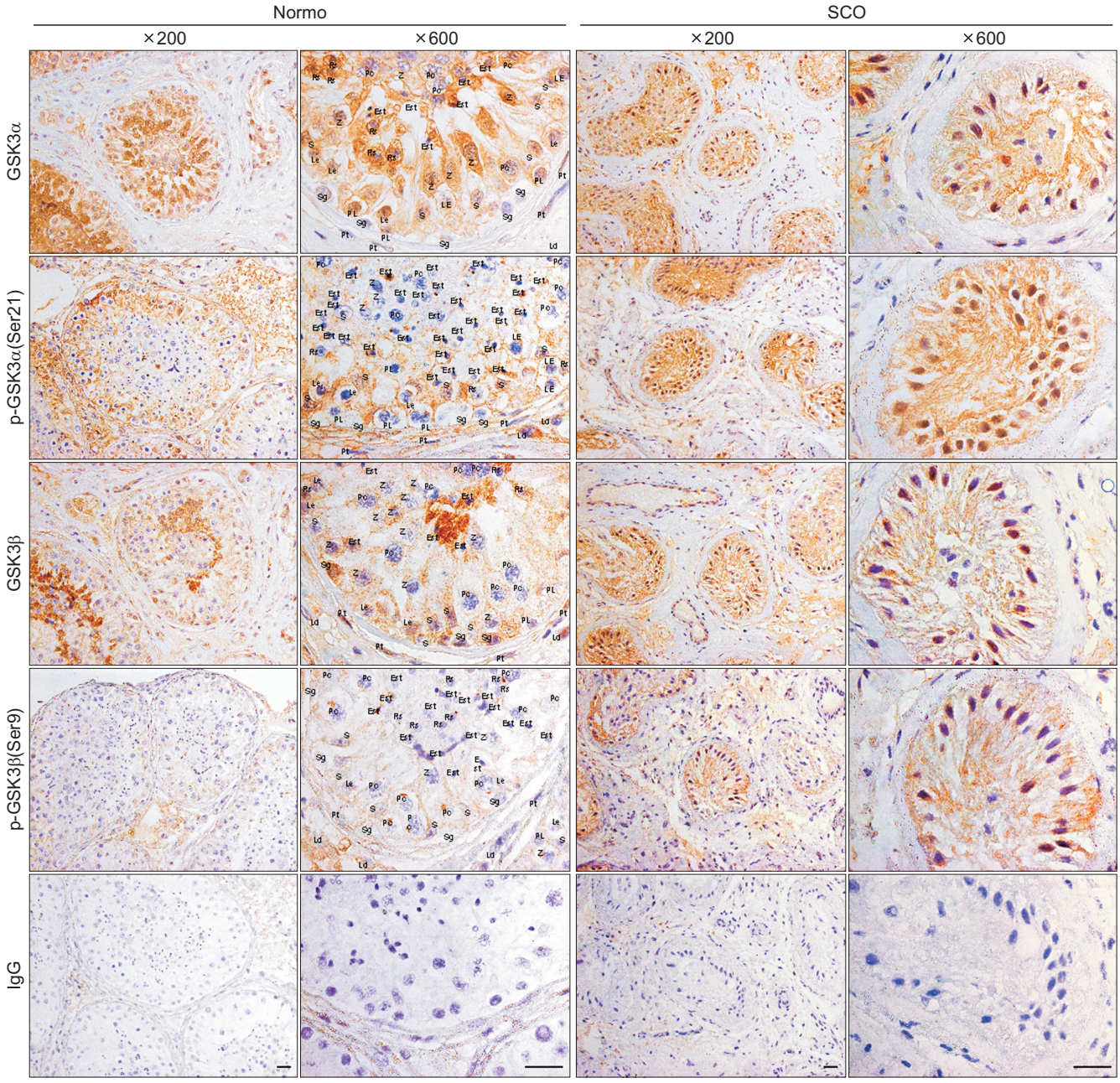

Fig. 3. Immunolocalization of (phospho-) GSK3 isoforms in human testes. Immunohistochemistry of GSK3α, GSK3β, p-GSK3α(Ser21), and p-GSK3β(Ser9) in human testis biopsies. In normospermatogenic testes, GSK3α was primarily localized in preleptotene spermatocytes (PL), leptotene spermatocytes (Le), zygotene spermatocytes (Z), pachytene spermatocytes (Pc), round spermatids (Rs), elongating/elongated spermatids (Est), and Sertoli cells (S), but it was weak to marginal in spermatogonia (Sg), peritubular cells (Pt), and Leydig cells (L). In contrast, p-GSK3α(Ser21) was primarily localized in S, Pt, and L but marginal in PL and Le. GSK3β immunoreactivity was primarily found in Sg, Rs, Est, S, Pt, and L but weak to marginal in PL and Pc. p-GSK3β(Ser9) was primarily localized in S and L and was weak to marginal in Pt, Sg, spermatocytes, and Est. In Sertoli cell-only (SCO) testes, GSK3α immunoreactivity was localized in S, L, and Pt. p-GSK3α(Ser21) immunoreactivity in S was increased compared to that of the normosperamtogenic testes. GSK3β immunoreactivity was localized in S, Pt, and L. p-GSK3β(Ser9) immunoreactivity in S was markedly increased compared to that of the normospermatogenic testes. Bar=20 μm.

visible immunoreactivity was found in negative control. Regardless of acrosome reaction, fluorescence intensity of GSK3α/β in head was significantly higher than those in midpiece and tail whereas p-GSK3α(Ser21) signals in midpiece and tail were significantly higher than those in head (Fig. 4).

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4. Phosphorylation of GSK3 isoforms in human spermatozoa

In a western blot of human sperm lysates, 51-kDa GSK3α was predominant and was phosphorylated at the Ser21 residue, whereas only a small amount of GSK3β was detected, and no visible p-GSK3β(Ser9) band was detected. p-GSK3α(Ser21) and p-Tyr (a capacitation marker) levels were visibly increased after incubation in TCM (Fig. 5A). In densitometric analysis, p-GSK3α(Ser21) levels were significantly increased after incubation in TCM (Fig. 5B). In CASA, the total and progressive motility of spermatozoa were increased after incubation in TCM (Fig. 5C).

DISCUSSION

1. GSK3 isoform variants in human spermatogenesis

In mammals, two transcript variants of *GSK3β* (*β1* and *β2*) have been reported [14,15]. In normospermatogenic testis, *GSK3α*, *GSK3β1*, and *GSK3β2* mRNAs were expressed. Of note, *GSK3β2* mRNA was more abundant than *GSK3β1* mRNA in the testes. GSK3β1, which contains a 13-amino-acid insert in the catalytic domain, has lower kinase activity than *GSK3β2* toward tau [14]. Given that GSK3β is primarily expressed in the spermatogonia, decreased *GSK3β2* mRNA in SCO testes is attributable to the lack of spermatogonia, and which could be a useful marker for spermatogo-

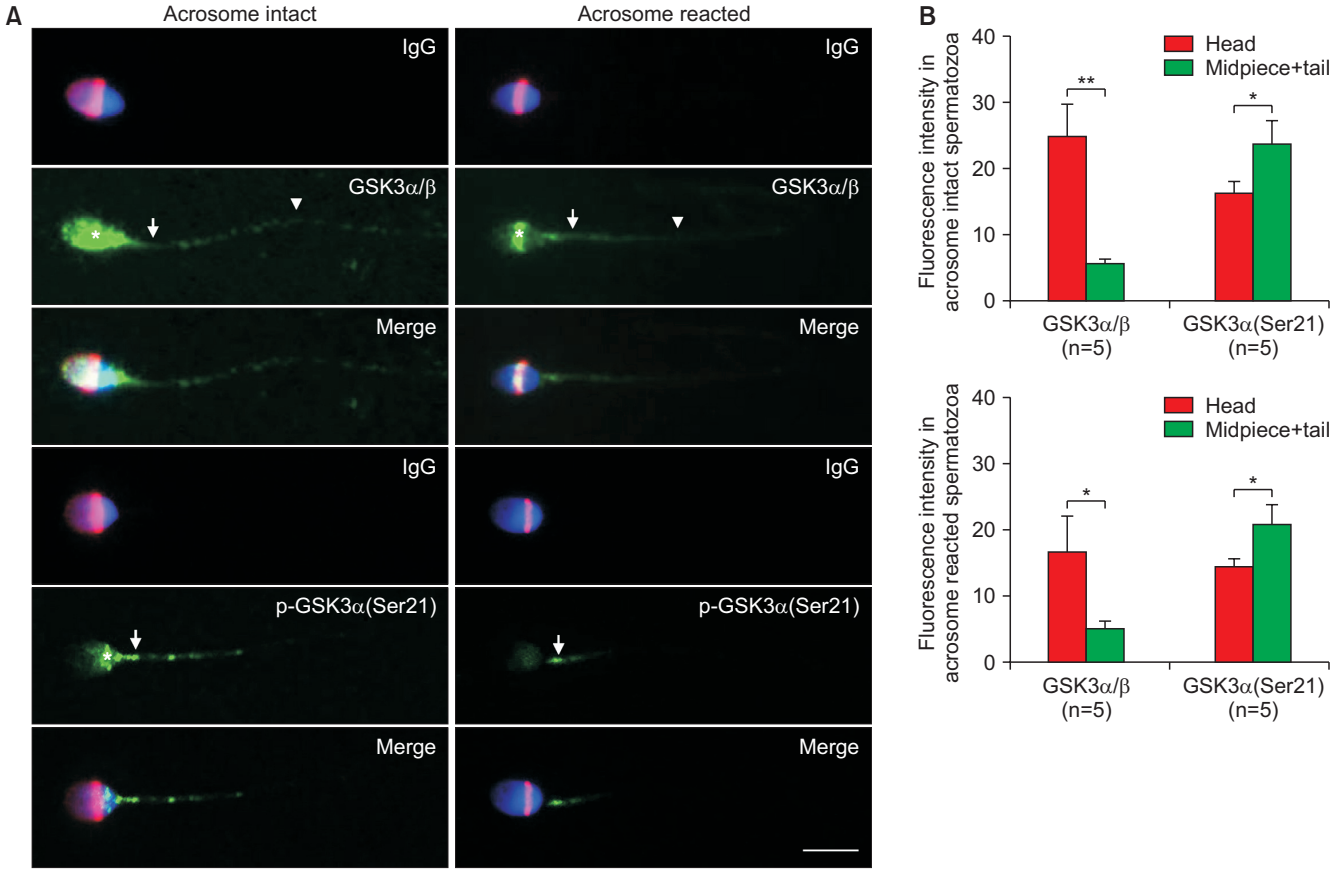

Fig. 4. Expression of GSK3 in human spermatozoa. (A) Immunocytochemistry of GSK3α/β and p-GSK3α(Ser21) in human spermatozoa. In acrosome-intact spermatozoa, the immunoreactivity of GSK3α/β was localized in the head, midpiece, and principal piece of spermatozoa (marked by asterisks, arrows, and arrowheads, respectively). The immunoreactivity of p-GSK3α(Ser21) was localized in the post-acrosomal region of the head and in the midpiece (marked by asterisks and arrows, respectively) of the tail. After acrosome reaction, the immunoreactivity of GSK3α/β disappeared in the acrosomal and post-acrosomal regions of the head and was only found in the equatorial region of the head and in the midpiece and principal piece of the tail (marked by asterisks, arrows, and arrowheads, respectively). The p-GSK3α(Ser21) was found in the midpiece of the tail (marked by arrows). GSK3α/β and p-GSK3α(Ser21) are represented by green signals. Nuclei were stained blue by DAPI. Bar=5 μm. (B) Subcellular fluorescence intensity of GSK3α/β and p-GSK3α(Ser21) in human spermatozoa. Regardless of acrosome reaction, relative fluorescence intensity of GSK3α/β in head was significantly higher than those in midpiece and tail. p-GSK3α(Ser21) signal in midpiece and tail was significantly higher than those in head. * and ** significantly different by Mann–Whitney U test at $p<0.05$ and <0.01 levels, respectively.

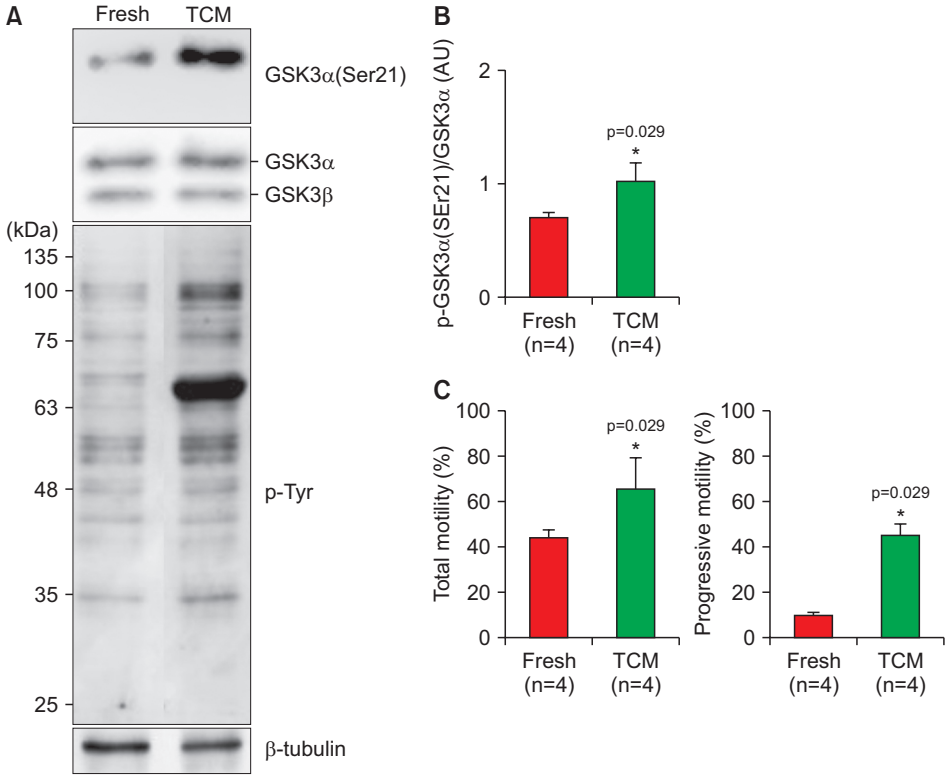

Fig. 5. Inhibitory phosphorylation of GSK3 in human spermatozoa during capacitation. (A) Western blots of p-GSK3α(Ser21) and phosphotyrosine (p-Tyr, a capacitation marker) proteins in human spermatozoa after incubation in Tyrode's complete medium (TCM). The p-GSK3α(Ser21) and p-Tyr levels were markedly increased after incubation in TCM for 3 hours. (B) Densitometric analysis of p-GSK3α(Ser21) in human spermatozoa after incubation in TCM for 3 hours. Inhibitory phosphorylation of GSK3α was significantly increased during capacitation (n=4). (C) Changes in total and progressive motility of human spermatozoa after incubation in TCM for 3 hours. Total and progressive motility were significantly increased during capacitation (n=4). Data are presented as mean+standard deviation. * significantly different from fresh spermatozoa by Mann–Whitney U test at $p<0.05$.

nial population in human testes. On western blot of testes, the 51-kDa form corresponding to GSK3α was predominant, and a small amount of the 46-kDa form corresponding to GSK3β2 was expressed. Given that tested monoclonal antibody is produced by immunizing animals with a 31 amino acid synthetic peptide surrounding 31 Gln269 of human GSK3α and GSK3β, GSK3β 1 and 3 might be not expressed or out of detection limit. In SCO testes, GSK3α levels were visibly lower than those of normospermatogenic testes. Given that GSK3α is abundant in meiotic and post-meiotic germ cells [16], the decreased GSK3α in SCO testes is attributable to the absence of developing germ cells.

2. Suggested role of GSK3 isoforms in spermatogenesis

In developing germ cells, GSK3β was primarily expressed in the spermatogonia and elongating/elongated spermatids whereas GSK3α was predominant in meiotic spermatocytes and round spermatids. Given that GSK3β is dominant in somatic tissues [17], the dominance of GSK3α is a unique feature of meiotic and post-meiotic germ cells including spermatozoa. LCRs are important for interaction between LCR-containing proteins and their interacting partners [18]. Although the kinase domain was conserved, the positions and lengths of the LCRs are quite different between GSK3α and β, implying that the interacting partners are quite different between the GSK3 isoforms. In spermatogonia, immunoreactivity of p-GSK3β(Ser9) was weak to marginal, indicating that the kinase activity of GSK3β is active. In mice, Wnt/β-catenin signaling which phosphorylates GSK3, stimulates proliferation of undifferentiated spermatogonia [19]. Although the role of GSK3β in the spermatogonia remains to be clarified, the kinase activity of GSK3β might be required for control of spermatogonial proliferation and differentiation in human testes. The abundance of GSK3α but p-GSK3α(Ser21) and GSK3β in early spermatocytes suggests the switching of GSK3β to GSK3α at meiotic entry. In spermatids expressing the GSK3α/β, p-GSK3α/β(Ser21/9) immunoreactivity was weak to negligible, suggesting that the kinase activity of GSK3 is quite high. GSK3 participates in the remodeling of cytoskeletons by phosphodegron-dependent protein degradation [20]. Therefore, the kinase activities of GSK3α/β in the post-meiotic male germ cells might be involved in the degradation of cellular structure during spermiogenesis [21]. In mice, the gene deletion of GSK3α decreased spermatogenesis and sperm fertility [9]. Although the isoform-specific roles of GSK3 in mitotic

spermatogonia and meiotic and post-meiotic germ cells remain to be elucidated, GSK3α could play a role in the meiosis as well as post-meiotic differentiation of human male germ cells. GSK3 is an important kinase involved in the regulation of cellular metabolism. Active GSK3 impedes glycolysis and suppress glycogen synthesis, decreasing the energy production [22]. Accumulation of glucose-6-phosphate decreases the inhibitory phosphorylation of GSK3, lowering the glycolysis [22,23]. Undifferentiated spermatogonia get energy primarily *via* glycolysis until the oxidative phosphorylation increases when the spermatogonia start to differentiate [24]. Therefore, weak expression of phospho-GSK3 in spermatogonia might be a reflection of low levels of glycolysis. In mice, isoform-specific functions of GSK3 in insulin sensitivity, glycogen synthase phosphorylation, and blood glucose levels have been known [25]. Together differentially expressed GSK3 isoforms may regulate different metabolic activity in developing male germ cells.

3. Role of GSK3α/β in Sertoli cells and Leydig cells

In the Sertoli cells of normospermatogenic testes, GSK3α/β were weakly phosphorylated on Ser21/9, suggesting that kinase activity of GSK3α/β is quite high in active Sertoli cells. Given that Sertoli cells do not proliferate during adulthood, the active GSK3α/β in Sertoli cells may participated in germ cell nutrition and tissue remodeling to support spermatogenesis. In the Sertoli cells of SCO testes, by contrast, GSK3α/β were heavily phosphorylated on Ser21 and Ser9, respectively. This suggests that the kinase activity of GSK3α/β is kept low in the inactive Sertoli cells of SCO testes. Sertoli cells in SCO testes showed dedifferentiated phenotype, resembling those in fetal testes [26,27]. The massive inhibitory phosphorylation of GSK3α/β and the correspondingly low kinase activity of GSK3 might be coupled with decreased cellular metabolism in the Sertoli cells of SCO testes devoid of spermatogenesis. In Leydig cells of normospermatogenic testes, GSK3α but GSK3β was heavily phosphorylated on Ser21. This suggests that kinase activity of GSK3β is high in Leydig cells. In mice Leydig cells, GSK3 inhibition stimulated steroidogenic activity [28]. Although GSK3 isoform specific role in the Leydig cells remains to be resolved, GSK3 may actively participate in steroidogenesis in Leydig cells of human testes.

4. Role of GSK3α/β in spermatozoa

In the PNA-positive, acrosome-intact human spermatozoa, GSK3α/β and p-GSK3α(Ser21) were expressed in the acrosome and post-acrosomal region of the head and the midpiece. In mammalian spermatozoa, GSK3 in the head could regulate acrosomal exocytosis by phosphorylating dynamin [29,30]. In goat spermatozoa, GSK3 in acrosomal region was associated with acrosome reaction [31]. BIO, an ATP-competitive GSK3 inhibitor, reduced spontaneous acrosome reaction in bovine spermatozoa [30]. Together, in the human spermatozoa, acrosomal GSK3 may participate in the acrosome reaction. In the acrosome-reacted spermatozoa, GSK3 was only found in the equatorial region and p-GSK3α(Ser21) in post-acrosomal regions was disappeared. Given that equatorial region of acrosome-reacted sperm fuses with oolemma, the kinase activity of GSK3α in the equatorial region may participate in the interaction between acrosome-reacted spermatozoa and oocyte. In the tail, p-GSK3α(Ser21) was found primarily in the midpiece. In mitochondria, GSK inhibition significantly reduced ATP consumption [32]. In boar spermatozoa, heat stress decreased sperm motility *via* the activation of GSK3α and the inhibition of mitochondrial protein import, which is rescued by a GSK3 inhibitor [18]. In human spermatozoa, GSK3α and p-GSK3α(Ser21) in the midpiece suggests the mitochondrial function of GSK3α. In mammalian spermatozoa, inhibitory phosphorylation on serine 21 residue of GSK3α has been suggested as key regulator of sperm motility [9,10]. Following the incubation of human spermatozoa in TCM, p-GSK3α(Ser21) and p-Tyr levels were increased together with motility, supporting that the inhibitory phosphorylation of GSK3α is important for capacitation and motility activation in human spermatozoa.

CONCLUSIONS

In conclusion, GSK3 isoforms switch from GSK3β to GSK3α during meiotic entry, suggesting the differential roles of GSK3α and GSK3β in in meiosis and stemness or mitotic proliferation of spermatogonia, respectively. In the inactive Sertoli cells of SCO testes, kinase activity of GSK3 is kept low *via* inhibitory phosphorylation. GSK3α in the head and midpiece of human spermatozoa may participate in acrosome reaction and motility activation *via* regulation of mitochon-

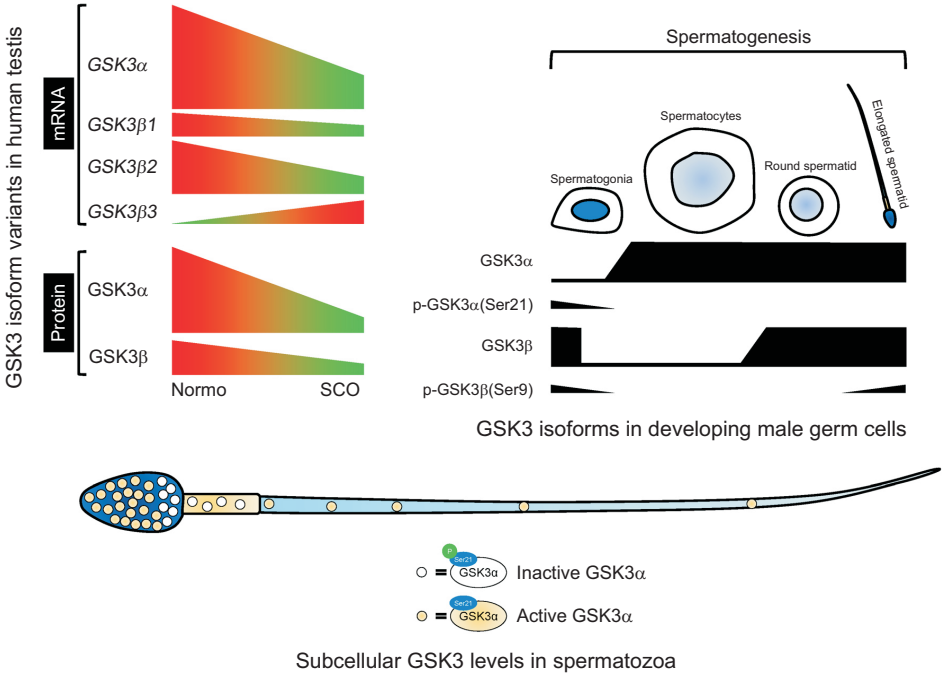

Fig. 6. GSK3 in human testes and spermatozoa. In Sertoli cell-only (SCO) testes, GSK3 proteins and *GSK3α*, *GSK3β1*, and *GSK3β2* mRNA were decreased but *GSK3β3* mRNA was increased compared to those in normospermatogenic testes. GSK3α was marginally expressed in spermatogonia but visibly increased in meiotic germ cells. GSK3β was highly expressed in spermatogonia and spermatids but marginal in spermatocytes. Inhibitory phosphorylation of GSK3α/β was kept low during spermatogenesis. In the acrosome-intact spermatozoa, GSK3α/β of was found in the head, midpiece, and tail. p-GSK3α(Ser21) was primarily expressed in the post-acrosomal region of the head, midpiece and tail.

drial function (Fig. 6).

Conflict of Interest

The authors have nothing to disclose.

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Author Contribution

Conceptualization: MCG, JTS. Data curation: SHP, YX. Formal analysis: SHP. Funding acquisition: MCG, JTS. Investigation: SHP, YX. Methodology: MCG, JTS, YSP. Project administration: MCG, JTS. Resources: JTS, YSP. Software: SHP, YX. Supervision: MCG. Validation: MCG, SHP, YX. Visualization: SHP. Writing – original draft: MCG, SHP, YX. Writing – review & editing: MCG, SHP.

Data Sharing Statement

The data analyzed for this study have been deposited in HARVARD Dataverse and are available at https://doi.org/10.7910/DVN/ZXQ3UN.

REFERENCES

1. Galliher-Beckley AJ, Williams JG, Collins JB, Cidlowski JA. Glycogen synthase kinase 3beta-mediated serine phosphorylation of the human glucocorticoid receptor redirects gene expression profiles. Mol Cell Biol 2008;28:7309-22.
2. da Silva RM, Noce BD, Waltero CF, Costa EP, de Abreu LA, Githaka NW, et al. Non-classical gluconeogenesis-dependent glucose metabolism in Rhipicephalus microplus embryonic cell line BME26. Int J Mol Sci 2015;16:1821-39.
3. Yu JS, Cui W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. Development 2016;143:3050-60.
4. Karlin S, Brocchieri L, Bergman A, Mrazek J, Gentles AJ. Amino acid runs in eukaryotic proteomes and disease associations. Proc Natl Acad Sci U S A 2002;99:333-8.
5. Bannen RM, Bingman CA, Phillips GN Jr. Effect of low-complexity regions on protein structure determination. J Struct Funct Genomics 2007;8:217-26.
6. Bowdish KS, Yuan HE, Mitchell AP. Analysis of RIM11, a yeast protein kinase that phosphorylates the meiotic activator IME1. Mol Cell Biol 1994;14:7909-19.
7. Moraveji SF, Attari F, Shahverdi A, Sepehri H, Farrokhi A, Hassani SN, et al. Inhibition of glycogen synthase kinase-3 promotes efficient derivation of pluripotent stem cells from neonatal mouse testis. Hum Reprod 2012;27:2312-24.

8. Zhu H, Liu C, Sun J, Li M, Hua J. Effect of GSK-3 inhibitor on the proliferation of multipotent male germ line stem cells (mGSCs) derived from goat testis. Theriogenology 2012;77:1939-50.
9. Bhattacharjee R, Goswami S, Dudiki T, Popkie AP, Phiel CJ, Kline D, et al. Targeted disruption of glycogen synthase kinase 3A (GSK3A) in mice affects sperm motility resulting in male infertility. Biol Reprod 2015;92:65.
10. Vijayaraghavan S, Mohan J, Gray H, Khatra B, Carr DW. A role for phosphorylation of glycogen synthase kinase-3alpha in bovine sperm motility regulation. Biol Reprod 2000;62:1647-54.
11. Gong Y, Guo H, Zhang Z, Zhou H, Zhao R, He B. Heat stress reduces sperm motility via activation of glycogen synthase kinase-3α and inhibition of mitochondrial protein import. Front Physiol 2017;8:718.
12. Nishimura T, Sakai M, Yonezawa H. Effects of valproic acid on fertility and reproductive organs in male rats. J Toxicol Sci 2000;25:85-93.
13. Verrotti A, Mencaroni E, Cofini M, Castagnino M, Leo A, Russo E, et al. Valproic acid metabolism and its consequences on sexual functions. Curr Drug Metab 2016;17:573-81.
14. Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J 1990;9:2431-8.
15. Mukai F, Ishiguro K, Sano Y, Fujita SC. Alternative splicing isoform of tau protein kinase I/glycogen synthase kinase 3beta. J Neurochem 2002;81:1073-83.
16. Bhattacharjee R, Goswami S, Dey S, Gangoda M, Brothag C, Eisa A, et al. Isoform-specific requirement for GSK3α in sperm for male fertility. Biol Reprod 2018;99:384-94.
17. Lau KF, Miller CC, Anderton BH, Shaw PC. Expression analysis of glycogen synthase kinase-3 in human tissues. J Pept Res 1999;54:85-91.
18. Coletta A, Pinney JW, Solís DY, Marsh J, Pettifer SR, Attwood TK. Low-complexity regions within protein sequences have position-dependent roles. BMC Syst Biol 2010;4:43.
19. Takase HM, Nusse R. Paracrine Wnt/β-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. Proc Natl Acad Sci U S A 2016;113:E1489-97.
20. Koivisto L, Häkkinen L, Matsumoto K, McCulloch CA, Yamada KM, Larjava H. Glycogen synthase kinase-3 regulates cytoskeleton and translocation of Rac1 in long cellular extensions of human keratinocytes. Exp Cell Res 2004;293:68-80.
21. Robertson H, Hayes JD, Sutherland C. A partnership with the proteasome; the destructive nature of GSK3. Biochem Pharmacol 2018;147:77-92.
22. Papadopoli D, Pollak M, Topisirovic I. The role of GSK3 in metabolic pathway perturbations in cancer. Biochim Biophys Acta Mol Cell Res 2021;1868:119059.
23. Rajas F, Gautier-Stein A, Mithieux G. Glucose-6 phosphate, a central hub for liver carbohydrate metabolism. Metabolites 2019;9:282.
24. Lord T, Nixon B. Metabolic changes accompanying spermatogonial stem cell differentiation. Dev Cell 2020;52:399-411.
25. MacAulay K, Doble BW, Patel S, Hansotia T, Sinclair EM, Drucker DJ, et al. Glycogen synthase kinase 3alpha-specific regulation of murine hepatic glycogen metabolism. Cell Metab 2007;6:329-37.
26. Nah WH, Lee JE, Park HJ, Park NC, Gye MC. Claudin-11 expression increased in spermatogenic defect in human testes. Fertil Steril 2011;95:385-8.
27. Nistal M, Gonzalez-Peramato P, De Miguel MP. Sertoli cell dedifferentiation in human cryptorchidism and gender reassignment shows similarities between fetal environmental and adult medical treatment estrogen and antiandrogen exposure. Reprod Toxicol 2013;42:172-9.
28. Gunosewoyo H, Midzak A, Gaisina IN, Sabath EV, Fedolak A, Hanania T, et al. Characterization of maleimide-based glycogen synthase kinase-3 (GSK-3) inhibitors as stimulators of steroidogenesis. J Med Chem 2013;56:5115-29.
29. Reid AT, Anderson AL, Roman SD, McLaughlin EA, McCluskey A, Robinson PJ, et al. Glycogen synthase kinase 3 regulates acrosomal exocytosis in mouse spermatozoa via dynamin phosphorylation. FASEB J 2015;29:2872-82.
30. Belenky M, Breitbart H. Role and regulation of glycogen synthase kinase-3 beta in bovine spermatozoa. Mol Reprod Dev 2017;84:8-18.
31. Zhu Z, Li R, Wang L, Zheng Y, Hoque SAM, Lv Y, et al. Glycogen synthase kinase-3 regulates sperm motility and acrosome reaction via affecting energy metabolism in goats. Front Physiol 2019;10:968.
32. Das S, Wong R, Rajapakse N, Murphy E, Steenbergen C. Glycogen synthase kinase 3 inhibition slows mitochondrial adenine nucleotide transport and regulates voltage-dependent anion channel phosphorylation. Circ Res 2008;103:983-91.