Isoform-specific GSK3A activity is negatively correlated with human sperm motility

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Submitted on December 6, 2018; resubmitted on January 29, 2019; editorial decision on February 18, 2019; accepted on February 19, 2019

In mouse and bovine sperm, GSK3 activity is inversely proportional to motility. Targeted disruption of the GSK3A gene in testis results in normal spermatogenesis, but mature sperm present a reduced motility, rendering male mice infertile. On the other hand, GSK3B testis-specific KO is fertile. Yet in human sperm, an isoform-specific correlation between GSK3A and sperm motility was never established. In order to analyze GSK3 function in human sperm motility, normospermic and asthenozoospermic samples from adult males were used to correlate GSK3 expression and activity levels with human sperm motility profiles. Moreover, testicular and sperm GSK3 interactomes were identified using a yeast two-hybrid screen and coimmunoprecipitation, respectively. An extensive *in-silico* analysis of the GSK3 interactome was performed. The results proved that inhibited GSK3A (serine phosphorylated) presents a significant strong positive correlation ($r = 0.822$, $P = 0.023$) with the percentage of progressive human sperm, whereas inhibited GSK3B is not significantly correlated with sperm motility ($r = 0.577$, $P = 0.175$). The importance of GSK3 in human sperm motility was further reinforced by *in-silico* analysis of the GSK3 interactome, which revealed a high level of involvement of GSK3 interactors in sperm motility-related functions. The limitation of techniques used for GSK3 interactome identification can be a drawback, since none completely mimics the physiological environment. Our findings prove that human sperm motility relies on isoform-specific functions of GSK3A within this cell. Given the reported relevance of GSK3 protein–protein interactions in sperm motility, we hypothesized that they stand as potential targets for male contraceptive strategies based on sperm motility modulation.

Key words: sperm motility / sperm biochemistry / interactome / GSK3

Introduction

Glycogen synthase kinase 3 (GSK3), a serine/threonine kinase, has been involved in a wide range of cellular processes such as apoptosis, mitosis and proliferation (Kaidanovich-Beilin and Woodgett, 2011; Beurel et al., 2015). Moreover, deregulation of GSK3 functions has been associated with pathological conditions such as cancer, Alzheimer's disease and diabetes (Amar et al., 2011; Gao et al., 2011). GSK3 is ubiquitously expressed and is encoded by two genes giving rise to two isoforms: GSK3A and GSK3B. The isoforms differ in their N-termini, with GSK3A having a unique glycine-rich N-terminus which is highly conserved in mammals, suggesting an isoform-specific function (Azoulay-Alfaguter et al., 2011).

GSK3 plays a central role in the male reproductive system. In mouse testis, GSK3A is expressed in the seminiferous tubules and its expression increases during the onset of spermatogenesis, peaking in the adult testis (Bhattacharjee et al., 2015). GSK3B expression is present in cells entering meiosis, spermatids and Sertoli cells (Guo et al., 2003). Curiously, targeted disruption of GSK3A gene in testis, results in normal spermatogenesis, but the mature sperm present a reduced motility and metabolism, rendering male mice infertile (Bhattacharjee et al., 2015, 2018). On the other hand, the GSK3B testis-specific knock-out was fertile (Bhattacharjee et al., 2018). In mouse and bovine sperm, GSK3 activity is inversely proportional to sperm motility and in immotile caput sperm, GSK3 activity is six times higher than that of motile caudal sperm (Vijayaraghavan et al., 1996, 2000). GSK3 activity

is controlled by its phosphorylation state. When serine phosphorylated, GSK3 catalytic activity is low (GSK3A Ser9 and GSK3B Ser21) but when tyrosine phosphorylated, it is activated (GSK3A Tyr 279 and GSK3B Tyr 216) (Wang et al., 1994).

Although in bovine and mouse, the role of GSK3 in male fertility is well established, in human sperm the knowledge is limited. With that in mind, we performed a GSK3 characterization, by determining its activity levels in asthenozoospermic and normozoospermic ejaculated human samples and its subcellular location in human sperm. The observation that mouse GSK3B cannot substitute for GSK3A implies that GSK3A is essential for normal sperm physiology. We considered that the unique role of GSK3A in sperm motility is reliant on its interactors and, as such, identified the GSK3A and GSK3B interactomes in both human testis and sperm.

Materials and Methods

Ethical approval

This study was approved by the Ethics and Internal Review Board of the Hospital Infante D. Pedro E.P.E. (Aveiro, Portugal) (Process number: 36/AO) and was conducted in accordance with the ethical standards of the Helsinki Declaration. All donors signed an informed consent forms allowing the samples to be used for scientific purposes.

All procedures using mice were performed at the Kent State University animal facility and were approved by the National Institute of Environmental Health Sciences institutional Animal Care and Use Committee (IACUC) and the Kent State Animal Ethics Committee under the IACUC protocol number 362DK 13-11. Immediately after CO₂ euthanasia, testis and epididymis of 3–4-month-old CD1 mice (*Mus musculus*) were removed.

Sperm extracts

Human ejaculate semen samples were obtained from healthy donors by masturbation into a sterile container. Basic semen analysis was performed by qualified technicians according to World Health Organization (WHO) guidelines (Organization, 2010). There was no significant presence of non-sperm cells in the sample (round cells $<1.0 \times 10^6$ cells/mL). After semen liquefaction, sperm cells were washed in phosphate buffered saline (PBS1x, Fisher Scientific, Loures, Portugal).

Immunoblotting

Washed human sperm were lysed in either: Tris buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA) (Fisher Scientific); 1xRIPA (0.05 M Tris-HCl, pH 7.4, 0.150 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) (Millipore Iberica, Madrid, Spain); 1xRIPA modified (0.05 M Tris-HCl, pH 7.4, 0.150 M NaCl, 0.25% deoxycholic acid, 2% NP-40, 1 mM EDTA); or 1%SDS (Fisher Scientific, Loures, Portugal), for 30 min on ice and centrifuged at 16 000 g, 15 min, 4°C. The supernatant was recovered (protein extract). Mouse sperm cells and testis were lysed in 1xRIPA, centrifuged at 16 000 g, 15 min, 4°C and the protein extract was recovered. Human testis protein extract was acquired from Takara, Enzifarma, Lisboa, Portugal (ref: 635 309).

Sperm protein extracts were mass normalized using BCA assay (ref: 23 225, Pierce, Fisher Scientific) separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. Afterwards, the membrane was incubated with one of the following antibodies: mouse anti-GSK3A/B (Invitrogen, Fisher Scientific, ref: 44-610, 1:2000) rabbit anti-GSK3A (Cell Signaling Technology, Danvers, MA, USA, ref: #9338, 1:1000) rabbit anti-GSK3B (Cell Signaling ref: #9315, 1:1000); mouse anti-GSK3A pS21 (Santa

Cruz Technologies, Heidelberg, Germany, ref: sc-365 483, 1:1000); mouse anti-GSK3B pS9 (Santa Cruz Technologies ref: sc-373 800, 1:1000) and rabbit anti-LRP6 (Cell Signaling, ref: #2560, 1:1000), 4°C, ON. Finally, the membrane was incubated with the appropriate infrared secondary antibody (1:5000, Li-Cor Biosciences UK Ltd, Cambridge, UK). The images were obtained using Odyssey Infrared Imaging Bands System (Li-Cor Biosciences). Bands were quantified with the Quantity One 1-D Analysis Software (Bio-Rad, Amadora, Portugal). Phosphoserine GSK3 levels were calculated by determining the ratio between phosphoserine signal and total GSK3 signal. GSK3 levels were normalized to the loading control Ponceau S. The statistical measures used were the mean and SEM. A test of normality (Shapiro-Wilk test) was performed to assess normality of quantitative variables. The Pearson correlation coefficient *r* was determined to assess the relationship between two variables. Statistical analysis was conducted using the Statistical Package for Social Sciences, version 19 (SPSS®, Chicago, IL, USA). The significance level was set at 0.05.

Immunocytochemistry

Washed human sperm were spread onto a glass coverslip, allowed to dry and fixed in 4% formaldehyde (Fisher Scientific) for 10 min. Afterwards, sperm were permeabilized in 0.1% Tween (Fisher Scientific) in 1% goat serum (Sigma-Aldrich Química, S.A., Sintra) and 5% BSA (NZYTech, Lisboa, Portugal) for 20 min. Blocking was performed with 1% goat serum and 5% BSA for 1 h 30 min and then incubated with primary antibodies: rabbit anti-GSK3A (Cell Signaling ref: #9338, 1:50) or rabbit anti-GSK3B (Cell Signaling ref: #9315, 1:50) overnight at 4°C; or rabbit anti-LRP6 (Cell Signaling, ref: #2560, 1:50), rabbit anti-pLRP6 1490 (Cell Signaling, ref: #2568, 1:50) or rabbit anti-AKAP11 (Invitrogen, Fisher Scientific, ref: PA5-39 868, 1:100) for 1 h 20 min. The sperm cells were incubated with anti-rabbit Alexa 594 nm (Life Technologies S.A., Madrid, Spain, 1:800) for 45 min at room temperature. Coverslips were washed in PBSx1 + 0.1% Tween, followed by one wash step in PBS1x. Finally, Hoechst was added, and coverslips were mounted onto a glass slide with ProLong™ Gold Antifade Mountant (Invitrogen, Fisher Scientific, ref: 10 144). Negative controls were processed in parallel. Fluorescence images were obtained using an Imager.Z1, Axio-Cam HRm camera and AxioVision software (Zeiss, Jena, Germany). Three normospermic human sperm samples were analyzed and around 100 cells per sample were assessed.

Yeast two-hybrid screen

Homo Sapiens GSK3A (NM_019 884.2) was subcloned using EcoRI and BamHI (New England Biolabs, Herts, UK) and Homo Sapiens GSK3B (NM_002 093.3) was subcloned using NdeI and SalI (New England Biolabs) into pAS2-I plasmid. Both vectors were sequenced to ensure that GSK3A and GSK3B were in frame with Gal-AD. The pAS2-I-GSK3A and pAS2-I-GSK3B vectors were transformed into AH109 yeast strain by a standard lithium acetate method (Clontech, Takara). GSK3A and GSK3B are not cytotoxic to AH109 yeast cells. Expression of GSK3A and GSK3B was confirmed and both proteins did not activate the reporter genes per se (Supplementary Fig. S1). For library screening, AH109 transformed with either pAS2-I-GSK3A or pAS2-I-GSK3B was mated with yeast strain Y187 expressing human testis cDNA library in pGADT7-Rec (Mate&Plate Library—Human testis ref. 630 470, Clontech, Takara) according to manufacture instructions. Half of the mating mixture was plated onto high-stringency medium (Quadruple dropout medium: SD/-Ade/-His/-Leu/-Trp) and the other half was placed onto low-stringency medium (Triple dropout medium: SD/-His/-Leu/-Trp) and the plates were incubated at 30°C. Colonies obtained in the low-stringency plates were replica plated onto medium with X-A-Gal and incubated at 30°C to check for MEL-I expression (blue color colonies).

For GSK3A, 93 positive clones were obtained from a total 2.64×10^7 screened clones. For GSK3B, 54 positive clones were obtained from a total of 2.75×10^7 screened clones. The Matchmaker Insert Check PCR mix 2 (Clontech, Takara, ref:630 497) was used to identify positive clone cDNA. DNA sequences were compared to the GeneBank database to identify the corresponding protein.

Coimmunoprecipitation

Sperm cells (50×10^6) were lysed in 1xRIPA, supplemented with 1 mM of Phenylmethylsulfonyl fluoride (PMSF) (Fisher Scientific) and 0.2 mM of sodium orthovanadate (Na_3VO_4) (Fisher Scientific) for 60 min on ice and centrifuged at 16 000 g, 4°C, 15 min. Sperm extracts were pre-cleared using Dynabeads Protein G (ref: 10 003D, Invitrogen, Fisher Scientific) and incubated with either rabbit anti-GSK3A (Cell Signaling ref: #9338, 1:50), rabbit anti-GSK3B (Cell Signaling ref: #9315, 1:50) or rabbit anti-IgG (ref: sc-2027, Santa Cruz Technologies) at 4°C ON with rotation. After incubation, 50 μL of dynabeads were added and incubated for 2 h. After washing with PBS1x, the dynabeads were resuspended in 50 mM glycine (Fisher Scientific) for 5 min. Finally, the supernatant was recovered and 1%SDS was added to the dynabeads, which were incubated 5 min, boiled and recovered.

Alternatively, GSK3 antibodies were crosslinked using BS3 (bis(sulfosuccinimidyl)suberate) (Invitrogen, Fisher Scientific, ref: 21 580) to Dynabeads Protein G, according to manufacturer instructions. Sperm extracts were pre-cleared and incubated with crosslinked beads for 1 h. After washing, the beads were resuspended in trypsin digestion buffer (20 mM Tris-HCl pH 8.0, 2 mM CaCl_2).

Mass spectrometry

Mass spectrometry studies of GSK3 human sperm interactors were performed in two facilities (two distinct samples).

In the Lerner Research Institute's Proteomics and Metabolomics Laboratory, the LC-MS system was a Dionex Ultimate 3000 nano-flow HPLC interfacing with a Finnigan Orbitrap LTQ Elite hybrid ion trap mass spectrometer system. The HPLC system used an Acclaim PepMap 100 precolumn ($75 \mu\text{m} \times 2 \text{ cm}$, C18, $3 \mu\text{m}$, 100 Å) followed by an Acclaim PepMap RSLC analytical column ($75 \mu\text{m} \times 15 \text{ cm}$, C18, $2 \mu\text{m}$, 100 Å). The data was analyzed by using all CID spectra collected in the experiment to search the human UniProtKB protein database with the search programs, Sequest and Mascot. Only results with mascot score $P < 0.05$ and at least two identifying peptides with mascot ion scores of at least 40 were considered. Specifically, GSK3A and GSK3B sequences searches were performed in the Sequest program.

In the VIB Proteomics Core Facility, the LC-MS-MS system was Ultimate 3000 RSLCnano system in-line connected to a Q Exactive mass spectrometer (Thermo, Fisher Scientific, Loures, Portugal). Peptides were loaded on a reverse-phase column (made in-house, $75 \mu\text{m}$ I.D. $\times$ 20 mm, $3 \mu\text{m}$ beads C18 Reprosil-Pur, Dr. Maisch). Each sample was injected three times and analyzed in triplicate. Data analysis was performed with MaxQuant (version 1.5.6.5) (Cox and Mann, 2008), using the Andromeda search engine on default search settings, including a false discovery rate of 1% at both the peptide and protein levels. The spectra were searched against human proteins in the UniProtKB database (release version January 2017), with only proteins containing at least one unique or razor peptide being retained. Proteins were quantified by the MaxLFQ algorithm integrated in the MaxQuant software (Cox et al., 2014), with quantification requiring a minimum ratio count of two unique or razor peptides. Further data analysis was performed with the Perseus software (version 1.5.5.3) (Tyanova et al., 2016) after uploading the protein groups file from MaxQuant. Proteins were identified by site, reverse database hits and contaminants were removed, and the technical replicate samples of GSK3A,

GSK3B and the negative control were grouped. Proteins with fewer than three valid values in at least one group were removed and the missing values were imputed from a normal distribution around the detection limit. Finally, t-tests were performed ($\text{FDR} = 0.0001$ and $S0 = 5$) to compare samples of GSK3A and GSK3B with the negative control (Silva et al., 2017).

In-silico analysis

Either UniProtKB or FASTA sequence was retrieved for all GSK3 interactors and used for subsequent *in-silico* analysis (only Homo sapiens information was considered). The presence of GSK3 consensus phosphorylation site (xxx[ST]xxx[ST]P) (Wu et al., 2009) was analyzed in: Eukaryotic Linear Motif (ELM) resource (Dinkel et al., 2016); PhosphoSitePlus (Hornbeck et al., 2015); Kinase Net (<http://www.kinaset.net>); NetPhos 3.1 Server (Blom et al., 2004); ScanProsite (de Castro et al., 2006); and GPS 3.0 (Xue et al., 2011). Only data obtained with high threshold, high conservation scores and reported in at least three tools were considered. GSK3A and GSK3B interactomes were retrieved from IMEx-curated databases (Orchard et al., 2012) and Human Integrated Protein-Protein Interaction rEference (HIPPIE) database (Alanis-Lobato et al., 2017). Only interactions for human GSK3A and GSK3B with human proteins were considered (March 2018).

Gene expression patterns (mRNA) for all interactors (whether identified in this study or obtained from databases) were retrieved from: The Human Protein Atlas (Uhlén et al., 2015); Pattern Gene Database (PaGenBase) (Pan et al., 2013) Expression atlas EMBL-EBI (68 FANTOM5 project-adult; 32 Uhlen's Lab and GTEx) (Petryszak et al., 2014); BioGPS (Wu et al., 2016); and UniGene (Pontius et al., 2003). mRNA expression values for all databases (Transcripts per million or fragments per kilobase of exon model per million mapped reads) were retrieved and testis expression values were normalized by calculating the percentage of testis expression taking into account the expression of all tissues. Only interactors that presented more than 50% of expression in testis in at least two databases used were considered highly expressed in testis and were classified in three categories: 50–75%, 75–90% and >90%. Differently expressed proteins in asthenozoospermic samples were collected from peer reviewed papers and compared with GSK3 interactors (Cai et al., 2006; Chen et al., 2009; An et al., 2011; Jing et al., 2011; Li et al., 2010, 2011; Shen et al., 2013b; Amaral et al., 2014; Bhagwat et al., 2014; Salvolini et al., 2014; Hashemitabar et al., 2015; Zhou et al., 2015; Saraswat et al., 2017).

Phenotypes associated with all interactors (genes) were retrieved from Mouse Genome Informatics (MGI) (Eppig et al., 2015) and OMIM (Amberger et al., 2015). Manually curated genes associated with phenotypes of male infertility retrieved from DisGeNet (Piñero et al., 2017), Phenopedia (Yu et al., 2010) and DISEASE database. Altered accessory glands, genetic disorders, sexual behavior and tumor incidence were excluded. Also, GSK3A and GSK3B interactors annotated to testis and sperm physiology on GeneOntology enrichment tool (PANTHER version 12.0, 25 August 2017) (Gene Ontology Consortium, 2015) were classified according to those annotations. GSK3A and GSK3B protein-protein interaction (PPIs) networks were built using Cytoscape v 3.6.0 (Shannon et al., 2003). The inner connections between those proteins were captured. To construct sperm motility and testis related GSK3A and GSK3B PPI networks, GSK3A and GSK3B interactors associated with sperm motility and testis phenotypes were extracted from GSK3A and GSK3B interactome network.

Statistical analysis

The statistical measures used were the mean and SEM. A test of normality (Shapiro-Wilk test) was performed to assess normality of quantitative

variables. The Pearson correlation coefficient, r , was determined to assess the relationship between two variables. Statistical analysis was conducted using the Statistical Package for Social Sciences, version 19 (SPSS®, Chicago, IL, USA). The significance level was set at 0.05.

Results

GSK3A is required for human progressive spermatozoa motility

Characterization of GSK3 in mouse, bovine and primates sperm is well established (Smith et al., 1999; Vijayaraghavan et al., 1996, 2000). Yet, in human spermatozoa, characterization of GSK3 is deficient. We evaluated the expression and activation of both GSK3 isoforms in human testis and ejaculated human sperm (Fig. 1).

Figure 1A shows that GSK3A and GSK3B were expressed in human testis and sperm, similar to what was previously described in mouse and bovine. The levels of inhibited GSK3A and GSK3B (serine phosphorylation) were assessed in human testis and sperm. In human testis, no serine phosphorylated GSK3 was detected, while in human sperm both phosphorylated GSK3 isoforms were detected (Fig. 1A). Moreover, different lysis-buffer strength recovered different amounts of GSK3 in human sperm (Supplementary Fig. S2).

To assess the correlation between GSK3 activity with ejaculated sperm motility, total and serine phosphorylated GSK3A and GSK3B (low activity) were evaluated in normospermic and asthenozoospermic samples (see Supplementary Table SI for sample information). Total GSK3A and serine phosphorylated GSK3A levels appear to be lower in asthenozoospermic samples compared to normospermic (Fig. 1B and C), while expression of total and serine phosphorylated GSK3B appeared to be similar in both normospermic and asthenozoospermic samples (Fig. 1B and C). Also, Fig. 1D shows that there was a significant strong positive correlation ($r = 0.822$, $P = 0.023$) between the percentage of progressive sperm and the levels of inhibited GSK3A, whereas inhibited GSK3B was not significantly correlated with sperm motility ($r = 0.577$, $P = 0.175$). Although the levels of total GSK3 were not significantly correlated with the percentage of progressive motility, GSK3A presented an apparent positive correlation with the percentage of progressive motile spermatozoa (Fig. 1E). The correlation between GSK3 levels and the percentage of immotile spermatozoa was also analyzed, and the results comply with that previous described (Supplementary Table SII).

GSK3A and GSK3B have distinct distributions in human spermatozoa

The subcellular localization of GSK3A and GSK3B in ejaculated human sperm was analyzed. Figure 2 shows that GSK3A was primarily located in the flagellum (98.0%) and 75.7% of sperm cells also showed immunoreactivity in the head. Curiously, 24.2% of the spermatozoa showed a strong immunoreactivity for GSK3A in the equatorial region, particularly at the edges. In contrast, GSK3B was mainly located in the sperm head (97.0%); 23.9% of sperm showed GSK3B distributed throughout the entire head and flagellum and in 76.0% of sperm cells, it was present only in the sperm head (Fig. 2). Within the flagellum, both GSK3A and GSK3B present a punctate like staining.

GSK3 human testis and sperm interactome

A yeast two-hybrid screen of a human cDNA testis library revealed 46 putative interactors for GSK3A and 21 for GSK3B (Supplementary Tables SIII and SIV, respectively). For GSK3A, 76% were new putative interactors while 24% were previously described as GSK3 interactors. Of GSK3A interactors identified, 58.7% contained the GSK3 consensus phosphorylation site (xxx[ST]xxx[ST]P). Finally, 34.8% of GSK3A interactors were already described to be present in either testis and/or sperm of mammals. For GSK3B, 77.8% were identified for the first time as GSK3B putative interactors. Around 38% of the GSK3B identified interactors had the GSK3 consensus phosphorylation site and 61.1% were previously reported to be present in testis and/or sperm of mammals.

GSK3A and GSK3B interactors were isolated from ejaculated human sperm by coimmunoprecipitation using isoform-specific GSK3 antibodies in two independent experiments by mass spectrometry analysis. Endogenous GSK3A and GSK3B were successfully immunoprecipitated in both experiments (Supplementary Fig. S3) and five GSK3A peptides and four GSK3B peptides were identified in mass spectrometry (Supplementary Table SV). Note that neither GSK3A nor GSK3B were detected in the negative control. There were 17 and 34 interactors identified as sperm GSK3A and GSK3B interactors, respectively (Supplementary Tables SVI and SVII, respectively). Regarding GSK3A interactors, 82.4% were potentially novel interactors and 17.6% contained the GSK3 consensus phosphorylation site. Also, 58.8% of GSK3A identified interactors were described as expressed in either mammalian testis and/or sperm by previous studies. For GSK3B interactors, 85.3% were new putative interactors and 47.1% were shown to be expressed in either mammalian testis and/or sperm. Finally, the GSK3 consensus phosphorylation site was present in 14.7% of GSK3B interactors.

GSK3 interactomes are associated with sperm motility and testis functions

To enrich the GSK3 human testis and spermatozoa interactome, GSK3A and for GSK3B interactomes were retrieved from public available databases; 75 GSK3A interactors and 413 GSK3B had been previously identified (Supplementary Tables SVIII and SIX, respectively). With the goal of identifying key GSK3 interactors for sperm and testis physiology, gene expression for GSK3 interactors was retrieved from five different tissue-expression databases (Supplementary Tables SVIII and SIX). Four GSK3A interactors are testis-enriched, with more than 90% of their expression restricted to testis: DDII1, GOLGA6C (testis interactors), ACR and PRSS37 (sperm interactors). Although not testis-enriched, TTC16 expression is enhanced in testis (Supplementary Table SX). For GSK3B interactors, besides ACR and PRSS37, TEK5, CMTM2 (testis interactors), HIST1H1T, PRKACG, TSKS (databases interactors) were classified as highly enriched in testis and CABYR expression is enhanced in testis (Supplementary Table SX). To further characterize the GSK3 interactome, differently expressed proteins in asthenozoospermic samples were retrieved from the proteomics studies. Overall, eleven GSK3 testis or sperm interactors are increased, eight are decreased and one has conflicting reports in asthenozoospermic samples (Supplementary Tables SIII, SIV, SVI and SVII).

PPI networks were constructed using data obtained from this study and retrieved from databases. The GSK3A interactome network

Figure 1 GSK3 in human testis and spermatozoa and correlation with sperm motility. **(A)** Western blot analysis of total GSK3 and serine phosphorylated GSK3 isoforms in human testis and spermatozoa, mouse testis and HeLa cells; 30 μ g of protein obtained in RIPA lysis were loaded per sample. From top to bottom, GSK3 was immunodetected with the following antibodies: anti-GSK3A/B antibody, anti-GSK3A antibody and anti-GSK3B antibody, anti-GSK3A pS21 and anti-GSK3B pS9. **(B)** Immunoblot of total and serine phosphorylated GSK3 isoforms in human normospermic ($n = 3$) and asthenozoospermic ($n = 4$) ejaculated spermatozoa. **(C)** Total and serine phosphorylated GSK3 isoforms protein levels in human normospermic and asthenozoospermic (bar chart with error bars (SEM)). **(D)** Pearson Correlation between the percentage of progressive motile ejaculated sperm and protein levels of serine phosphorylated GSK3A and GSK3B (scatter plot with regression line). **(E)** Pearson Correlation between the percentage of progressive motile ejaculated sperm and protein levels of total GSK3A and GSK3B. Blots were cropped. *Correlation is statistically significant at the 0.05 level.

(Supplementary Fig. S4) presents 130 proteins, including GSK3A. Between GSK3A interactors, 257 interactions are formed. The GSK3B interactome is composed by 456 proteins that form 1813 interactions among them (data not shown).

To add biological meaning to the GSK3 interactome, phenotype and Gene Ontology information were retrieved (Supplementary Tables SXI and SXII). Here, two subnetworks were extracted: sperm motility-related and testis-related GSK3-based networks. Of the GSK3A

interactors, 26 have been associated with motility-related functions, phenotypes and/or subcellular locations (Fig. 3). From those, five (PRSS37, DRC1, RPS19, HSPA5 and AP3D1) were identified in this study as GSK3A interactors and only one was classified as testis-enriched protein (PRSS37). PRKACA and DRC1 stand out by presenting five motility-related annotations followed by GLI3 with four such annotations. Note that GSK3A itself has been associated with locomotion and cell motility processes. For GSK3B, 100 interactors are

Figure 2 Subcellular localization of GSK3A and GSK3B in normospermic ejaculated human sperm. GSK3A is located in the flagellum (star) and head (arrowhead), more specifically in the equatorial region. GSK3B is located through the entire head (arrowhead) and occasionally in the flagellum (star). Per sample, 100 sperm cells were counted. The experiment was done in triplicate. Scale bar is 5 μm . Nucleus is marked in blue. ROI: region of interest. All images were obtained with 63 $\times$ magnification.

Figure 3 GSK3-centered subnetwork for sperm motility extracted from GSK3 interactome network. All GSK3 interactors associated with motility-related annotations were used to build the network. Solid lines: testis or sperm GSK3 interactions. Dashed lines: database-retrieved GSK3 interactions. Node size: according to testis expression. Node colors: represent motility-related phenotypes, biological processes (BP) or cellular components (CC).

annotated to motility-related categories, and of those, six are highly expressed in testis (CABYR, TEK5, PRKACG, PRSS37, TSKS and CMTM2) (Fig. 3).

Analyzing the GSK3 testis subnetwork (Fig. 4), 10 GSK3A interactors were associated with testis-related annotations and 2 of those were identified in this study (PRSS37 and HSP90AA1). Only PRSS37 was described as highly expressed in testis. With five testis-related annotations, we highlight AKAP9 and AR. For testis-related categories, GSK3B presents 45 interactors related to testis-related categories (Fig. 4).

Although not directly related to sperm motility and testis function, several GSK3 interactors are associated with more general annotations linked to the male reproductive system (Supplementary Fig. S5).

AKAP11 and LRP6 subcellular localization in human sperm

Two GSK3 interactors identified in this study, A-kinase anchor protein 11 (AKAP11) and low-density lipoprotein receptor-related protein 6 (LRP6), were chosen for further characterization, since they have been

previously implicated in the regulation of male fertility. Figure 5A shows that LRP6 is present in human testis and sperm at 180 kDa, the expected molecular weight of the protein. The band at the higher molecular weight could be due to post-translational modification, such as phosphorylation and N-glycosylation, known to occur in LRP6 (Khan et al., 2007; Niehrs and Shen, 2010). Immunocytochemistry studies (Fig. 5B), revealed that total LRP6 is localized to the entire length of the flagellum and occasionally at the post-acrosomal area. However, phosphorylated LRP6 (p1490LRP6) is restricted to the mid-piece. Moreover, a closer analysis showed that not all sperm cells present immunoreactivity towards LRP6 and p1490LRP6. Only 18 and 29% of sperm cells present immunoreactivity for LRP6 and p1490LRP6, respectively (Fig. 5B). Regarding AKAP11, this protein is localized on the anterior portion of the head and the equatorial area of ejaculated human sperm (Fig. 5B).

Discussion

GSK3 has been long associated with sperm motility acquisition and maintenance in mammals (Smith et al., 1999; Vijayaraghavan et al., 2000; Somanath et al., 2004). Recently, an isoform-specific function of

GSK3A in mice sperm primary motility acquisition has been suggested. However, the characterization of both GSK3 isoforms (GSK3A and GSK3B) in human sperm and testis physiology has been sparse. Assessment of a similar isoform-specific function of GSK3A in human sperm motility acquisition was thus necessary. This work aimed to characterize GSK3 isoforms in human sperm as well as identify and analyze the GSK3A and GSK3B interactome in human sperm and testis. Ultimately, this can help decipher the role of isoform-specific functions of GSK3 in human sperm physiology.

In this study we provided evidence to support that mature human sperm cells are unique in their need for GSK3A isoform to achieve progressive motility. Similar to other mammals, both GSK3 isoforms are present in human testis and sperm (Fig. 1). Characterization of GSK3 levels in normospermic and asthenozoospermic samples proved that in ejaculated human sperm, serine phosphorylated GSK3A presents a strong positive correlation with progressive sperm motility, but serine phosphorylated GSK3B does not show any correlation with sperm motility (Fig. 1). Furthermore, correlation between the percentage of immotile spermatozoa (Supplementary Table SII) and levels of serine phosphorylated GSK3A is in accordance with the correlation observed with progressive motile spermatozoa. This shows that

Figure 5 LRP6 and AKAP11 in human testis and ejaculated normospermic sperm. **(A)** Western blot analysis of LRP6 in human testis and ejaculated sperm, mouse testis and HeLa cells. For human testis, mouse testis and HeLa cells 30 μ g of protein were loaded per sample. For ejaculated human sperm, 100 μ g of proteins were loaded. Note according to the antibody datasheet, the antibody only recognizes human and rat LRP6. Arrow highlights the LRP6 presence in human sperm. **(B)** Subcellular localization of LRP6, p1490LRP6 and AKAP11 in mature human sperm. Total LRP6 is located in the entire flagellum (star) and occasionally in the post-acrosomal area (arrowhead). The phosphorylated form of LRP6 at serine 1490 is restricted to the midpiece (circle). AKAP11 is located to the head, specifically to the anterior and equatorial area (plus sign). Blots were cropped; 100 sperm cells were counted per samples. The experiment done in triplicate. Scale bar is 10 μ m. Nucleus is marked in blue. ROI, region of interest. All images were obtained with 63x magnification.

GSK3A activity is strongly correlated with human sperm motility, being a negative modulator, while GSK3B appears does not influence sperm motility. This is the first observation that GSK3 activity is associated with human sperm motility and that this function is a GSK3A isoform-specific function. The power of the study was limited by the relatively small samples size (three normozoospermic samples and four

asthenozoospermic samples). However, recent studies reinforce the results obtained. Using, knock-out technology a GSK3A isoform-specific function in mice sperm motility has been proved by Bhattacharjee and colleagues (Bhattacharjee *et al.*, 2015, 2018).

A possible explanation for the inability of GSK3B to substitute for GSK3A in human sperm relies on a distinct spatial expression pattern

between GSK3 isoforms in human sperm cells. The immunocytochemistry studies performed, showed that GSK3A is consistently present in the tail, but in only 75% of the cases was it also present in the head. Opposing GSK3A, GSK3B is always present on the head of human sperm, while its localization on the tail is irregular. A more plausible explanation for isoform-specific function of GSK3 in human sperm is GSK3 isoform-specific interactors that bind, target and modulate each isoform in human sperm. Work by Zeidner *et al.*, showed that RACK1 is a GSK3A isoform-specific interactor in the central nervous system, and that this interaction requires the unique glycine-rich GSK3A N-termini (Zeidner *et al.*, 2011).

With the purpose of identifying GSK3 isoform-specific interactions in the male reproductive system, the GSK3A and GSK3B interactomes in human testis and sperm were identified and characterized (Supplementary Tables III, IV, VI and VII). Due to technical restraints (human testis availability and sperm physiology), the human testis GSK3 interactome was constructed using an yeast two-hybrid approach, while for the human sperm GSK3 interactome, coimmunoprecipitation followed by mass spectrometry was undertaken. The yeast-two hybrid system relies on the yeast cell environment, not fully mimicking mammalian cells. Yet, it is one of the only techniques that indicates binary interactions. Coimmunoprecipitation does not prove direct interaction, and weak interactions are usually lost but retains intracellular environment conditions. Approximately 27% of the GSK3 interactions identified in this study have been previously described, which results in high confidence in the GSK3 interactomes identified. Furthermore, the interaction between GSK3 and AXIN2 was acknowledged, for the first time, in testis. This interaction is extensively described in somatic cells (Stamos and Weis, 2013; Voronkov and Krauss, 2013; Song *et al.*, 2014; Pronobis *et al.*, 2015), reinforcing our confidence in the results obtained for the GSK3 interactome.

With the purpose of constructing the most complete GSK3 interactome, GSK3 interactions available on PPIs databases were retrieved and GSK3-centered networks were constructed (Figs 3 and 4, Supplementary Figs S4 and S5). To identify GSK3 interactions key for sperm physiology (more specifically sperm motility), tissue-expression, phenotypes and gene ontology annotations were integrated into the GSK3 networks. It may be noted that the knowledge of protein tissue expression is still limited and typically does not take into account tissue-specific alternatively spliced transcripts. This is particularly relevant for testis, since testis is a tissue with a higher number of alternative transcripts splice variants (Elliott and Grellscheid, 2006; Uhlén *et al.*, 2015). None of GSK3A interactors listed on databases showed a testis-specific or -enriched expression. We identified the first testis-enriched or -specific GSK3A interactors which reflects the importance for deepening the knowledge on sperm physiology of our results. Regarding GSK3A interactions, 20.1% have a motility-related annotation and for GSK3B, 21% of interactors have been previously link to cell motility. This is relevant considering the sperm cells are the only human cells that possesses a progressive motile function. The fact that almost a quarter of the GSK3 interactome may be involved in the motility cell function reflects that the molecular mechanisms that control sperm cell motility are still partially unknown. Focusing on GSK3A interactions that have previously been shown to be involved, we highlight PRSS37. This protein is the only GSK3A interactor that is known to be associated with sperm motility, testis annotations, and categorized as enriched in testis (Fig. 3). Previous studies demonstrated that

when PRSS37 is absent in mice testis, fertilization is compromised due to inadequate spermatogenesis, decreased sperm oviduct-migration and decreased sperm-zona binding (Shen *et al.*, 2013a). Concerning testis functions, our analysis revealed that AR, PPP1CC and AKAP9 appear to have a prominent role since germ cells and other types of testicular cells are greatly affected by their absence (Fig. 4). These findings are in accordance with former studies (Varmuza *et al.*, 1999; Wang *et al.*, 2009; Schimenti *et al.*, 2013).

LRP6 and AKAP11, two GSK3 interactors identified in this study, were chosen for further characterization, as being previously involved in male reproduction. LRP6 was already described as involved in sperm motility and testis physiology, and AKAP11 in mouse spermatogenesis (Reinton *et al.*, 2000; Koch *et al.*, 2015). While the interaction between AKAP11 and GSK3B has been previously described (Tanji *et al.*, 2002), to our knowledge this is the first description of the interaction with GSK3A and the first time the interaction is described in human testis. In contrast with earlier studies in human sperm (Reinton *et al.*, 2000), we showed that AKAP11 is localized in the anterior portion of the head and equatorial region (Fig. 5B). In somatic cells, AKAP11 has been associated with cell migration (Logue *et al.*, 2011) and in 2002, Tanji *et al.* showed that AKAP11, PPP1, PRKACA and GSK3B formed a multimeric complex in which PPP1 and PRKACA controlled GSK3B activity. Since both PPP1 and PRKACA have been extensively described in mammalian testis and sperm (Smith *et al.*, 1996; Davidson *et al.*, 2005; Schimenti *et al.*, 2013), we may assume that similar multimeric complex may be formed to control GSK3 activity.

Bioinformatics analysis revealed that in mice, absence of LRP6 correlates with male infertility (Supplementary Fig. S5 and Supplementary Table SXI). Expression studies showed that LRP6 is present in human testis and sperm and is localized along the entire flagellum (Fig. 5A and B). Furthermore, when LRP6 is phosphorylated on S1490 (in somatic cells a GSK3 substrate (Davidson *et al.*, 2005; Zeng *et al.*, 2005)), its subcellular location is restricted to the human sperm midpiece (Fig. 5B). This is in accordance to earlier studies in mice and bovine sperm (Koch *et al.*, 2015). Interestingly, only a small percentage of the sperm cells within the same sample present staining for both LRP6 and S140 phosphorylated LRP6. Further studies to understand why this expression pattern occurs may prove useful. Koch *et al.* (2015) explored the non-genetic effects of B-catenin signaling on human sperm and suggested that the interaction between LRP6 and GSK3 is required for protein stabilization and consequently sperm motility. Therefore, despite the fact that in somatic cells GSK3/LRP6 interaction is involved in gene expression, our results reinforce that in human sperm this interaction can be fundamental for sperm physiology.

In conclusion, our data revealed an isoform-specific need for GSK3A in human progressive sperm motility modulation. The GSK3 interactome identified in this work uncovers the extent to which GSK3 can be involved in sperm motility and reveals new potential players in the molecular mechanisms of sperm motility. Even more, study of GSK3A interactors such as PRSS37 deserve to be pursued since the likelihood of this protein being involved in sperm motility is high. Furthermore, although we attempted to identify specific GSK3A interactors, no interaction was validated as GSK3A-unique. Selective GSK3A inhibitors and identification of specific targets of GSK3A can facilitate the development of a new group of male contraceptives based on sperm motility arrest.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Acknowledgements

We would like to thank Dr Phiel for providing the original vector containing the GSK3A and GSK3B ORF.

Authors' roles

M.J.F. designed the study, performed experiments, acquired, analyzed and interpreted the data and produced the article. J.V.S. performed experiments, interpreted the data and drafted the article. C.B. performed experiments and analyzed the data. B.R.C. analyzed and interpreted the data (statistical analysis). M.F. and S.V. designed the study and analyzed and interpreted the data. All authors critically revised the article and approved the final version.

Funding

FEDER funds through the 'Programa Operacional Competitividade e Internacionalização—COMPETE 2020' and by National Funds through the FCT—Fundação para a Ciência e Tecnologia (PTDB/BBB-BQB/3804/2014). We are thankful to Institute for Biomedicine—iBiMED (UID/BIM/04501/2013 and POCI-01-0145-FEDER-007628) for supporting this project. iBiMED is supported by the Portuguese Foundation for Science and Technology (FCT), Compete2020 and FEDER fund. Also, this worked was financed by the NIH grant R15 HD068971-01. Image acquisition was performed in the LiM facility of iBiMED, a node of PPBI (Portuguese Platform of Biolmaging): POCI-01-0145-FEDER-022122. This work was also supported by an individual grant from FCT of the Portuguese Ministry of Science and Higher Education to M.J.F. (SFRH/BD/84876/2012).

Conflict of interest

The authors declare that there are no conflicts of interest.

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