Novel expression of zona pellucida 3 protein in normal testis; potential functional implications

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ABSTRACT

The expression of the zona pellucida glycoprotein 3 (ZP3), originally thought to be specific for oocytes, was recently extended to ovarian, prostate, colorectal and lung cancers. Earlier successful ZP3 immunization of a transgenic mouse model carrying a ZP3 positive ovarian tumor emphasized the suitability of ZP3 for cancer immunotherapy. This study was carried out to determine whether any other normal tissues besides the ovary in healthy human and mouse tissues may express ZP3, considered important to exclude off-target effects of ZP3 cancer immunotherapy. Strong ZP3 expression was found in normal human and mouse testis. ZP3 protein and mRNA transcripts were localized in spermatogonia, spermatocytes and round and elongated spermatids of both human and mouse testis, as well as in a mouse spermatogonial cell line, but absent in testicular Sertoli, Leydig, spermatogonial stem and progenitor cells. All other normal human and mouse tissues were ZP3 negative. This surprising testicular ZP3 expression has implications for the development of ZP3 cancer immunotherapies, and it also alludes to the potential of using ZP3 as a target for the development of a male immunocontraceptive.

1. Introduction

The extracellular layer of the zona pellucida (ZP) surrounds mammalian oocytes (Wassarman 1999). It plays a crucial role in oogenesis, fertilization, prevention of polyspermy and embryo protection at the early stages of development (Wassarman 1999). ZP is composed of three or four glycoproteins that form a characteristic fibro-granular structure around the oocytes by non-covalent interactions (Wassarman and Mortillo 1991). In humans, ZP is composed of all the four glycoproteins named ZP1, ZP2, ZP3, and ZP4, whereas in mice only ZP1, ZP2, ZP3 are expressed (Harris et al., 1994; Lefievre et al., 2004).

ZP3 is essential for successful fertilization and plays a key role in the species-specific spermatozoa-oocyte binding and activation of the acrosome reaction (Bleil and Wassarman 1983; Wassarman 1999). ZP3 knockout female mice are infertile (Liu et al., 1996). Recent studies demonstrated that missense mutations in the ZP3 sequence in ZP-layer caused empty follicle syndrome in humans (Chen et al., 2017; Zhou et al., 2019). It has been suggested that ZP3 plays an important role during germinal vesicle breakdown in the early stage of meiosis and in further oocyte maturation (Gao et al., 2017). Higher ZP3 levels have been found in immature oocytes compared to mature ones (Canosa et al., 2017). However, there are very limited data on the ZP3 function other than its classical role as a component of the ZP.

Due to its critical role in the fertilization process, ZP3 became a

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natural candidate for female immunocontraception (Shabanowitz 1990), and has already been successfully used as a target antigen for active immunization of certain wild-life populations (Millar et al., 1989; Gupta et al., 1997). However, the loss of ovarian function caused by autoimmune oophoritis mediated by ZP3-specific antibodies and autoreactive T cells in various animal models (Mahi-Brown et al., 1988; Paterson et al., 1998) has precluded further contraceptive development in humans. Recently, a careful selection of epitope combinations has been found to induce infertility in animal models without any ovarian pathologies (Paterson et al., 2002; Mohd-Lila et al., 2019).

Despite the great interest in ZP3, there is a distinct shortage of studies investigating its potential expression outside the ovary. Recently, ectopic ZP3 expression was demonstrated in ovarian granulosa and epithelial cell cancers (Rahman et al., 2012), in transgenic mice presenting with granulosa cell tumors and in cell lines derived from these tumors as well as in human cancer cell lines (KK-1, KGN). ZP3 expression has also been shown in prostate, colorectal and lung cancers (Buschmann et al., 2004; Bennink 2016, Costa et al., 2018; Coelingh Bennink and RahmanInventor, 2019). Any future clinical cancer development with ZP3 immunization would require the characterization of the ZP3 expression profile in normal tissues. For this reason, we found it important to investigate the potential extraovarian ZP3 expression profile in healthy human and wild-type mouse tissues.

2. Materials and methods

2.1. Human tissue samples

We examined 94 normal human tissue samples (subjects age 30–45 years) from archival paraffin blocks or frozen tissues from the Medical University of Bialystok Department of Pathomorphology and the University Biobank, in Poland (n = 10/ovary; n = 8/testis; n = 7/myometrium smooth muscle; n = 7/skeletal muscle; n = 6/prostate and epididymis and n = 5/small intestine, large intestine, liver, pancreas, stomach, lymph node, brain, lung, kidney and breast for each tissue type). Samples were taken during the surgery and checked by the pathologist to ensure that they were healthy tissues and devoid of any cancer cells. The human testes samples removed during the surgery (n = 6), were received from the Medical University of Bialystok Biobank. None of the male patients had any case of infertility or spermatogenic maturation arrest. Testicular structure and spermatogonia, spermatocytes and spermatids were identified and distinguished based on the following characteristics: 1) typical size of the nuclear structure; 2) position in the epithelium; 3) typical pachytene morphology; 4) chromatin condensation patterns. Human semen samples (n = 6) were obtained from male patients treated for couple infertility problems (diagnosed female factors) recruited from the IVF program at the Department of Reproduction and Gynecological Endocrinology of the Medical University of Bialystok, Poland. Semen samples were obtained by masturbation after 3 days of sexual abstinence. After liquefaction at room temperature for 30–60 min, semen parameters were assessed according to the WHO guidelines (WHO 2010) and samples were used for the IVF procedure. The remaining samples were used to collect spermatozoa using the swim-up method. Samples with less than 15×10^6 spermatozoa/ml were excluded from this study. Written informed consent was obtained from all patients before inclusion. The local Human Investigations Ethics Committee at the Medical University of Bialystok, Bialystok Poland approved the study.

2.2. Mouse tissues samples

We tested a total of n = 40 normal 3 month-old wild-type C57BL mouse fresh or fixed tissue samples from paraffin blocks (n = 10 for ovary, testis, spleen and skeletal muscle each).

2.3. Cell cultures

The murine spermatogenic GC-2(spd)ts (CRL-2196; ATCC, Manassas, VA), murine Leydig cell tumor (BLTK-1) (Kananen et al., 1996) and Mouse Sertoli Cell line-1 (MSC-1) (Rebois 1982) cell lines (ATCC) were cultured in DMEM/F12 medium (GIBCO, Paisley, UK) supplemented with 10% (GC-2(spd)ts and BLTK-1) or 5% (MSC-1) fetal bovine serum (FBS; Biochrom, Berlin, Germany), 100 units/mL penicillin and 100 μg/mL streptomycin (P/S solution; Sigma-Aldrich, Saint Louis, MO) at 37 °C in a humidified atmosphere in the presence of 5% CO_2. The primary human Sertoli cell line HSerC (#4520; ScienCell Research Laboratories, Carlsbad, CA) was cultured in Sertoli Cell Medium (SerCM, #4521; ScienCell Research Laboratories), and the primary human Leydig cell line HLC (#4510; ScienCell Research Laboratories) in Leydig Cell Medium (LCM; #4511; ScienCell Research Laboratories) at 37 °C in a humidified atmosphere in the presence of 5% CO_2. Three independent cell platings were performed in triplicates for each RNA isolation and immunocytochemistry study.

2.4. RNA isolation

The TRIzol-based extraction method was used for total RNA isolation (Invitrogen, Carlsbad, CA). The quantity and quality of extracted RNA were determined by the absorbance measurement using NanoDrop (Thermo Scientific, Waltham, MA). Gel electrophoresis was performed to determine the integrity of isolated RNA.

2.5. RT-PCR analysis

Before the reverse transcription (RT) reaction, 1 μg of total RNA was incubated for 30 min with deoxyribonuclease (DNase I) (Invitrogen) at room temperature and then inhibited by 25 mM EDTA solution for 10 min at 65 °C. The RT reaction was performed with SensiFAST cDNA SynthesisKit (Bioline Reagents Ltd, London, UK) according to the manufacturer's protocol. First-strand cDNA was used as template in PCR (initial denaturation of 96 °C for 3 min, then 35 cycles of 94 °C for 1 min, 57 °C for 45 s, and 72 °C for 45 s, with a final extension period of 5 min at 72 °C). Primer sequences were as follows: mouse *Zp3* gene F: GAGCTTTTCGGCATTTCAAG, R: AGCTTATCGGGGATCTGGTT and mouse *Ppia* gene F: CATCCTAAAGCATACAGGTCCTG, R: TCCATGGCTTCCACAATGTT; human *ZP3* gene F: ATGCAGGTAACTGACGATGC, R: CCATCAGACGCAGAGAGAAA, human *FSHR* gene F: TGGGCTCAGGATGTCATCATCGGA, R: TGGATGACTCGAAGCTTGGTGAGG, human *LHR* gene F: CTGAGTGGCTGGGACTATGA, R: CCAAATCAGGACCCTAAGGA; and human *PPIA* gene F: GCCAAGACTGAGTGGTTGGATG, R: GAGTTGTCCACAGTCAGCAATGG.

2.6. Real-time quantitative PCR (qPCR)

For Real-time qPCR, SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) and the thermo-cycler 7500 Real-Time PCR System (Applied Biosystems) were used. Reaction conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C, and 1 min at 60 °C up to 40 amplification cycles. The PCR products were analyzed by melting curve analysis and agarose gel electrophoresis to ensure the amplification of a single product. Every reaction product (both for RT-PCR and qPCR) was separated and verified by sequencing analysis. Expression levels of the investigated genes were normalized to the housekeeping gene peptidylprolyl isomerase A (PPIA). The primer sequences were as follow: mouse *Zp3* F: *CCAACGACCAGACTGTGGAA*, R: *AGGACTATAGCTGCCAGGGT*; mouse *Ppia* F: *CATACAGGTCCTGGCATCTTGTC*, R: *AGACCACATGCTTGCCATCCAG*; and human *ZP3* F: TGGCAACAGCATGCAGGTA, R: CTGAGTGGCTGGGACTATGA, human *FSHR* F: GCCAAGAGAGCAAGGTGACA, R: CTCGAAGCTTGGTGAGGACA, human *LHR* F: CCGGTCTCACTCGACTATCACT, R: AAGCTTGAGATGGGATCACTTTG, and human *PPIA* F:

GTTCTTCGACATTGCCGTCG, R: TGTCTGCAAACAGCTCAAAGG.

2.7. RNAscope in situ hybridization

Formalin-fixed paraffin-embedded tissue samples were handled according to the manufacturer's protocol using RNAscope 2.0 HD Assay (catalog number 310033, Advanced Cell Diagnostics [ACD], Hayward, CA). In brief, slides were deparaffinized in xylene (2 × 5 min), 100% EtOH (2 × 1 min) and air-dried for 5 min at room temperature. Each section was treated with hydrogen peroxide for 10 min at RT, then washed twice in distilled water. Slides were boiled in antigen retrieval buffer for 15 min and submerged in distilled water immediately thereafter. Next, slides were washed in 100% EtOH and air-dried. For each section, barriers were drawn with the hydrophobic pen and protease was applied for 30 min in 40 °C in HybEZ™ Oven (ACD). Slides were washed twice in distilled water. Probes for the targeted transcripts (mouse ZP3: ACD-447551, human ZP3: ACD-442631) were applied as well as probes for positive (cyclophilin B–PPIB, a housekeeping gene, human ACD-313901; mouse ACD-313911) and negative controls (DapB - negative control probe targeting bacteria gene, ACD-310043). Then, slides were incubated at 40 °C for 2 h in the oven and washed 2 × 2 min in the wash buffer. Thereafter, hybridization amplifiers (AMPs) were applied for 30 min (AMP 1, 3, 5) or 15 min (AMP 2, 4, 6) at 40 °C (AMP 1–4) or at room temperature (AMP 5 and 6) with double washing in between every step. After the last washing, equal volumes of BROWN-A and BROWN-B reagents were combined and applied onto the sections for 10 min at RT. After double washing with distilled water, slides were counterstained in 50% Gill's hematoxylin (Vector Laboratories, Burlingame, CA, USA) for 2 min, then washed in 0.02% ammonia water for 10 s and twice in distilled water. Dehydrated slides (2 × 2 min in 70% EtOH, 2 × 2 min in 100% EtOH and 5 min in xylene) were mounted with Pertex (Histolab Products, Göteborg, Sweden).

2.8. Immunohistochemical staining

Monoclonal antibodies specific to human (Isotype IgG1 kappa) and mouse (Isotype IgG2a) ZP3 were produced with hybridoma techniques (East et al., 1985; Rankin et al., 1998). The hybridomas (ATCC® CRL-2462™ and ATCC® CRL-2569™) were cultured in CELLine bioreactor 1000 mL suspension flasks (Argos Technologies – Cole-Parmer, Vernon Hills, IL) in DMEM medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 4.5 g/L glucose supplemented with 10 mM HEPES, 0.15 mg/ml oxaloacetate, 0.05 mg/ml pyruvate, 0.0082 mg/mL bovine insulin and 0.05 mM 2-mercaptoethanol. The 20% FBS was implemented in the cell compartment and 1% FBS in the nutrient medium. Cells were harvested every ~5 days, depending on their growth and then the harvests were purified.

Formalin- or Bouin -fixed paraffin-embedded samples were deparaffinized and hydrated. Slides were boiled for 15 min in antigen retrieval buffer (10 mM citric acid buffer with 0.05% Tween20, pH = 6). Then, the sections were incubated in a humidified chamber for 1 h at RT with 3% BSA for reducing nonspecific background staining. Next, slides were incubated overnight in a humidified chamber at 4 °C with the primary antibody anti-ZP3 (0.125 μg/mL) or anti-vimentin (ab28028; Abcam, Cambridge, UK; dilution 1:300). Endogenous peroxidase activity was blocked by incubating slides in 0,5% H_2O_2 in PBS at room temperature for 20 min. DAKO polymer (DAKO EnVision + System – HRP labeled polymer; Agilent, Santa Clara, CA) was applied onto each section and incubated in a humidified chamber for 30 min at room temperature. DAB + Chromogen (DAKO) was applied for 5 min. Slides were washed in dH_2O, counterstained in Mayer's hematoxylin (Sigma-Aldrich, Saint Louis, MO), dehydrated and mounted with Pertex (Histolab Products). As a control for the antibodies, tissues were incubated with 3% BSA and DAKO polymer to differentiate unspecific from specific staining.

2.9. Immunocytochemical staining

GC-2(spd)ts cells cultured on collagen-coated microscope slide coverslips were washed twice with PBS and fixed in 3.7% paraformaldehyde (PFA) for 20 min. For spermatozoa staining, sperm smear was made and allowed to air-dry at RT for a minimum of 1 h. Next, slides with spermatozoa were fixed in 4% PFA in PBS for 1 h. Slides were rinsed in PBS and treated with 0.5% Triton X-100 in PBS for 15 min. To block unspecific binding sites, cells were incubated in blocking solution (2% BSA in PBS with 0.05% Tween 20). Thereafter, cells were incubated for 1 h with primary mouse monoclonal anti-ZP3 antibody or for dual staining anti-ZP3 antibody with anti- α-tubulin antibody (ab52866, Abcam; dilution 1:300) diluted in blocking solution. After washing, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (A11029; Thermo Fisher, Waltham, MA; dilution 1:500) or Alexa Fluor 488 goat anti-rabbit IgG (A11034; Thermo Fisher; dilution 1:500) and Alexa Fluor 546 goat anti-mouse IgG (A11030; Thermo Fisher; dilution 1:500) for 45 min. DAPI dye was used as a counterstain to detect cell nuclei. As a control for the antibodies, the cells were incubated with either 2% BSA or Alexa Fluor-488 goat anti-mouse IgG as a primary antibody to differentiate unspecific from specific staining.

2.10. Whole-mount mouse seminiferous tubule staining

Whole-mount staining of adult mouse seminiferous tubules was performed as previously described (Makela et al., 2020). Briefly, three adult WT male mice were sacrificed and the tubules were fixed with 4% PFA for 2 h at +4 °C. After blocking (2% BSA + 10% FBS in 0.3% Triton X-100 in PBS) the tubules were incubated overnight at +4 °C with primary antibodies: mouse monoclonal anti-human-ZP3 antibody (1 μg/ml), anti-GFRa1 antibody (AF560, R&D Systems, Minneapolis, MN; dilution 1:250) and anti-SALL4 antibody (1:2000, ab29112, Cambridge, UK). Following washes, the tubules were incubated with the respective secondary antibodies (A11055, A10036 and A31573, Life Technologies, Carlsbad, CA, USA, dilution 1:500 for all) for 1 h at RT. Finally, the tubules were ordered into liner strips, mounted and imaged (Zeiss LSM880, Carl Zeiss, Jena, Germany).

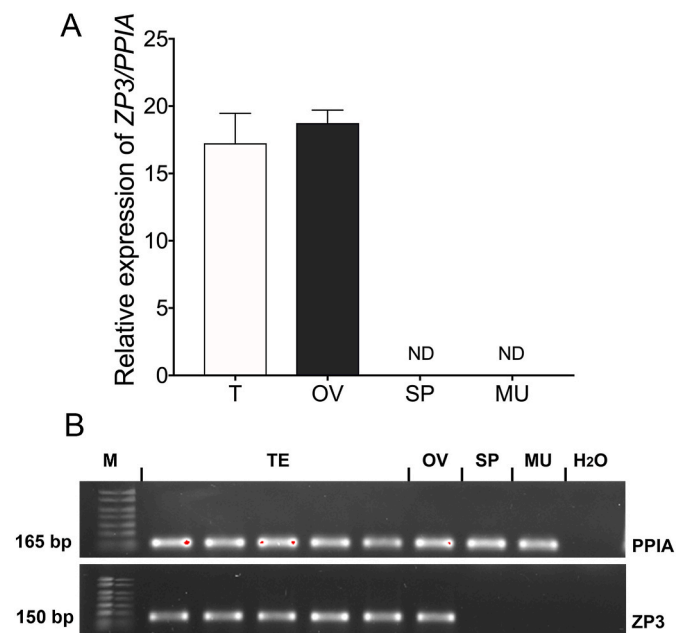## 2.11. Immunofluorescence staining of FFPE sections

PFA-fixed paraffin-embedded samples were deparaffinized and hydrated. Slides were boiled for 15 min in antigen retrieval buffer (10 mM citric acid buffer with 0.05% Tween20, pH = 6) and cooled down. Slides were washed three times in PBST for 5 min. The autofluorescence was blocked with 100 mM NH_4Cl for 3 min at RT in a humidified chamber, then the slides were washed three times in PBST for 5 min. Sections were incubated in a humidified chamber for 1 h at RT with a solution of combined 5% NGS and 1% BSA to reduce nonspecific background staining. Next, sections were incubated in a humidified chamber for 1,5 h at RT with the mix of anti-vimentin (1:1000; ab92547, Abcam, Cambridge, UK) and anti-ZP3 (1 μg/ml) primary antibodies suspended in PBST with 1% NGS and 0,5% BSA and washed three times in PBST for 5 min. After washing, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (A11029; Thermo Fisher, Waltham, MA; dilution 1:500) and Alexa Fluor 594 goat anti-rabbit IgG (A11012; Thermo Fisher; dilution 1:500) for 1,5 h at RT. DAPI dye was used as a counterstain to detect cell nuclei. As a control for the antibodies, the cells were incubated with either 2% BSA or Alexa Fluor-488 goat anti-mouse IgG as a primary antibody to differentiate unspecific from specific staining.

3. Results

3.1. Apart from the ovary, ZP3 is also expressed in normal human and wild-type (WT) mouse testis in spermatogenic cells

Quantitative PCR analysis showed *Zp3* expression in WT mouse

ovary and testis (Fig. 1A – 1B, Supplementary Table S1). Zp3 expression was not detectable in the WT mouse spleen and muscle used as negative controls (Fig. 1A–B). Immunohistochemical staining and RNAscope *in situ* hybridization localized abundant Zp3 protein and mRNA transcripts in oocytes from primary, secondary and antral follicles of the mouse ovary (Fig. 2A and C, respectively). Similarly, abundant Zp3 protein and mRNA were localized in spermatogonia, spermatocytes, round and elongated spermatids in the mouse testis (Fig. 2B and D, respectively). The mouse primordial follicle oocytes and testicular Sertoli and Leydig cells, as well as the spermatozoa, did not express Zp3 (Fig. 2A–D). In humans, abundant ZP3 protein and mRNA transcripts were also localized in the oocytes from ovarian follicles (Fig. 3A and C, respectively) and in testicular spermatogonia, spermatocytes, round and elongated spermatids (Fig. 3B and D, respectively). ZP3 expression was not detected in the human testicular Sertoli and Leydig cells, either at mRNA or protein levels (Fig. 3B and D). *Cyclophilin B* was used as a positive control, which was expressed abundantly in mouse and human ovary and testis (Supplemental Fig. 1, left column). *DapB* was used as a negative control, and it was not detected in any of the gonadal samples (Supplemental Fig. 1, right column). As a control for immunohistochemical analysis, the samples were incubated with a blocking solution with an omission of the primary antibody (Supplemental Fig. 2A and B) as well as with non-immune isotype control (IgG2A) for the primary anti-ZP3 antibody (Supplemental Fig. 3A and B), and no staining in mouse or human testis was detected. To identify Sertoli cells we immunostained testicular tissue with vimentin, a Sertoli cell marker. We observed positive immunoreactivity for vimentin in the cytoplasm of Sertoli cells with a thin layer of the cytoplasm passing through the whole epithelium (Supplemental Figs. 4A–B). We performed double immunostaining with vimentin and ZP3 to confirm that Sertoli cells did not express ZP3 (Supplemental Fig. 5). *ZP3* mRNA transcripts were not found in human primordial follicle oocytes (Supplemental Fig. 6A), whereas abundant expression was localized in oocytes from primary (Supplemental Fig. 6B), secondary (Supplemental Fig. 6C) and antral (Supplemental Fig. 6D) follicles. *Zp3* expression was also found in the

mouse GC-2spd(ts) immortalized spermatogenic cell line and WT mouse ovary as a positive control (Fig. 4A). No *Zp3* expression was detected in Leydig tumor cell line BLTK-1 and Sertoli MSC-1 cells (Fig. 4A). WT mouse skeletal muscle was used as *Zp3* negative control (Fig. 4A). Immunocytochemical studies localized Zp3 expression in the cytoplasms of mouse GC-2spd(ts) cells (Fig. 4B). No staining was detected in the control analysis without a primary antibody (Fig. 4B). *ZP3* mRNA expression was not detectable in either human primary Sertoli (HSerC) and Leydig (HLC) cells (Fig. 5A – 5B). *FSHR* and *LHR* expression were analyzed as a control for HSerC and HLC cells, respectively. HSerC expressed *FSHR* and no *LHR*, whereas HLC expressed *LHR* and no *FSHR* (Fig. 5A–B). *ZP3*, *LHR*, *FSHR* expression were found in human ovary used as a positive control, whereas was not detectable in human muscle used as a negative control (Fig. 5A–B). GFRa1-positive (GDNF family receptor alpha-1) spermatogonia are considered stem and progenitor cells (SSPCs) (differentiating spermatogonia, cells that precede A1 spermatogonia that are differentiated and committed to sperm development) of the male germline and thus responsible for the life-long sperm production (Makela and Hobbs 2019). In order to check Zp3 expression in SSPCs, we performed a whole-mount seminiferous tubule staining for adult mouse testis. We did not observe immunoreactivity for mouse monoclonal anti-ZP3 antibody on the basement membrane of adult mouse seminiferous epithelium suggesting that GFRa1/SALL4-positive (Spalt-like 4, a pan-spermatogonial marker (Chan et al., 2017)) SSPCs and differentiating progenitor spermatogonia (GFRa1-negative/SALL4-positive) do not express ZP3 (Fig. 6). Neither was *ZP3* mRNA expression detectable in human spermatozoa (Supplemental Fig. 7). *ZP3* expression was found in the human ovary used as a positive control, whereas it was not detectable in human muscle used as a negative control (Supplemental Fig. 7A). No ZP3 staining was detected using immunocytochemical analysis in human spermatozoa (Supplemental Fig. 7B).

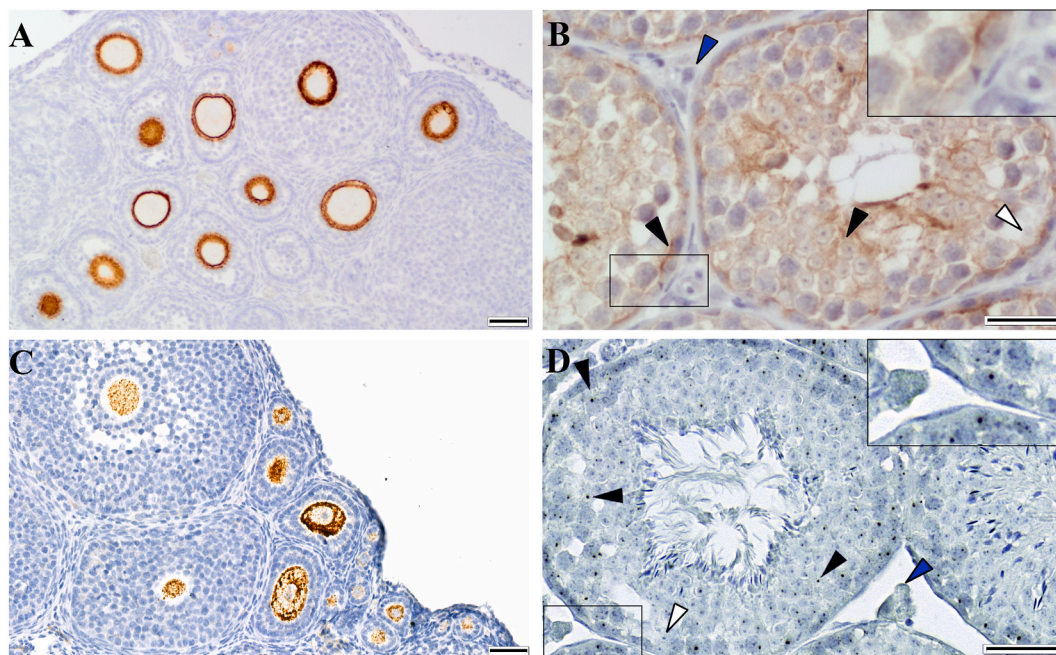### 3.2. Various other normal human and mouse tissues were ZP3 negative

ZP3 expression was not detected by immunohistochemical staining in several other normal human tissues, such as the small intestine (Supplemental Fig. 8a), large intestine (Supplemental Fig. 8b), liver (Supplemental Fig. 8c), pancreas (Supplemental Fig. 8d), stomach (Supplemental Fig. 8e), lymph node (Supplemental Fig. 8f), brain (Supplemental Fig. 8g), lung (Supplemental Fig. 8h), epididymis (Supplemental Fig. 9i), prostate (Supplemental Fig. 9j), kidney (Supplemental Fig. 9k), breast (Supplemental Fig. 9l), smooth muscle myometrium (Supplemental Fig. 9m) and skeletal muscle (Supplemental Fig. 9n).

4. Discussion

ZP3 has been shown to be expressed in several cancers (ovarian, prostate, colon and lung) (Buschmann et al., 2004; Rahman et al., 2012; Costa et al., 2018; Coelingh Bennink and RahmanInventor, 2019) as well as in ovarian cancer metastases and recurrences (Rahman et al., 2012), giving rise to the concept of its use as a target for cancer immunotherapy. We have provided the first evidence that ZP3 immunization is an effective treatment of ovarian cancer, using a transgenic mouse model of ovarian granulosa cell tumors (Rahman et al., 2012). The immunization strategy used has the potential of being effective in the treatment of other cancer types expressing ZP3. We designed the present study to characterize ZP3 expression in healthy tissues, with the potential of off-target effects in cancer immunization.

Our results showed the novel expression of ZP3 in spermatogonia, spermatocytes and spermatids in the human and mouse testis. However, no expression was found in mature spermatozoa, spermatogonial stem and progenitor cells and in Sertoli or Leydig cells. In the ovary, ZP3 is required for successful oocyte fertilization, but there is no data on any ZP3 function outside the ovary. High-throughput screening data from

Fig. 1. *Zp3* expression profile in different wild-type (WT) mouse tissues. The qPCR analysis of *Zp3* expression in normal mouse testis, ovary, spleen and muscle (A). Expression of *Zp3* was normalized to that of *Ppia* expression in the same sample. The RT-PCR analysis in 2% agarose gel of housekeeping gene *Ppia* and *Zp3* in normal mouse testis, ovary, spleen and muscle (B). Amplicon sizes are presented on the left. M, marker; MU, muscle; OV, ovary; SP, spleen; TE, testis; H_2O, nuclease-free water.

Fig. 2. Immunohistochemical localization and RNAscope *in situ* hybridization of Zp3 in wild-type (WT) mouse ovary and testis. Localization of Zp3 protein in mouse ovary (A) and testis (B) and *Zp3* mRNA transcripts in mouse ovary (C) and testis (D). The upper box on the right shows higher magnification of the lower box, showing *ZP3* protein/transcripts localization in mouse testis (B, D). Black arrow-heads show the positive ZP3 protein staining or single transcripts localization, white arrow-head indicates negative Sertoli cells and blue arrow-head shows negative Leydig cells in mouse testis. Please see all positive and negative controls for RNAscope *in situ* hybridization in Supplemental Fig. S1, immunohistochemistry in Supplemental Fig. S2 and all negative tissues in Supplemental Figs. 8 and 9. Scale bar, 100 μm (A, C) and 50 μm (B, D).
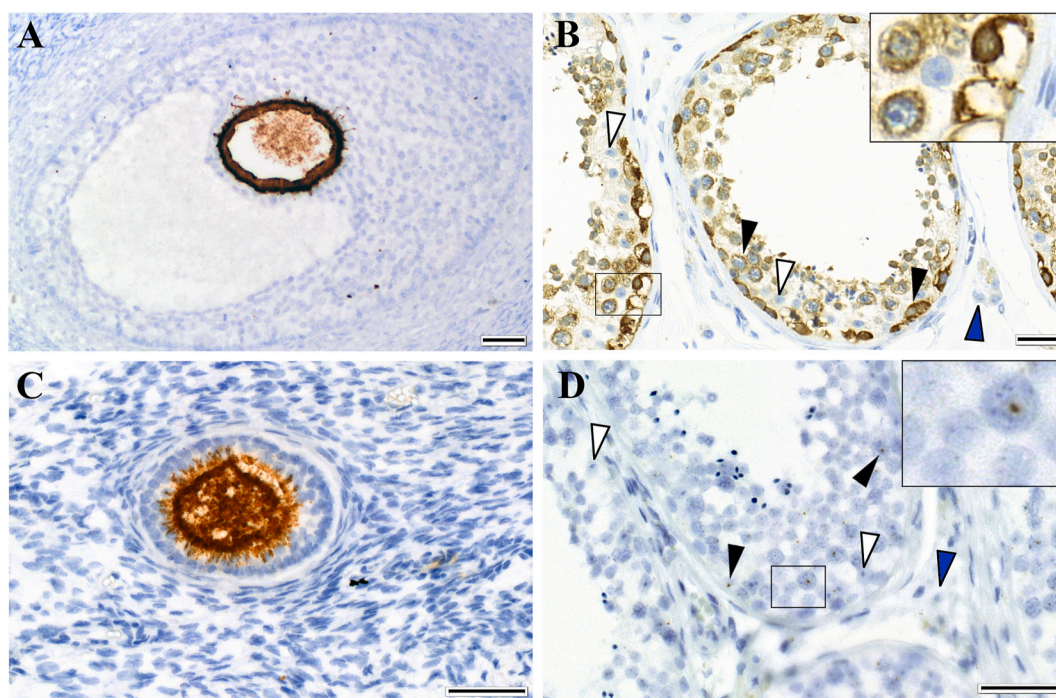
Fig. 3. Immunohistochemical localization and RNAscope *in situ* hybridization of ZP3 mRNA transcripts in normal human ovary and testis. Localization of ZP3 protein in the human ovary (A) and testis (B) and ZP3 mRNA transcripts in the human ovary (C) and testis (D). The upper box on the right shows higher magnification of the lower box, showing *ZP3* protein/transcripts localization in human testis (B, D). Black arrow-heads show the positive ZP3 protein staining or single transcripts localization, white arrow-head indicates negative Sertoli cells and blue arrow-head shows negative Leydig cells in human testis. Please see all positive and negative controls for RNAscope *in situ* hybridization in Supplemental Fig. S1, immunohistochemistry in Supplemental Fig. S2 and all negative tissues in Supplemental Figs. 8 and 9. Scale bar, 100 μm (A, B) and 50 μm (C, D).
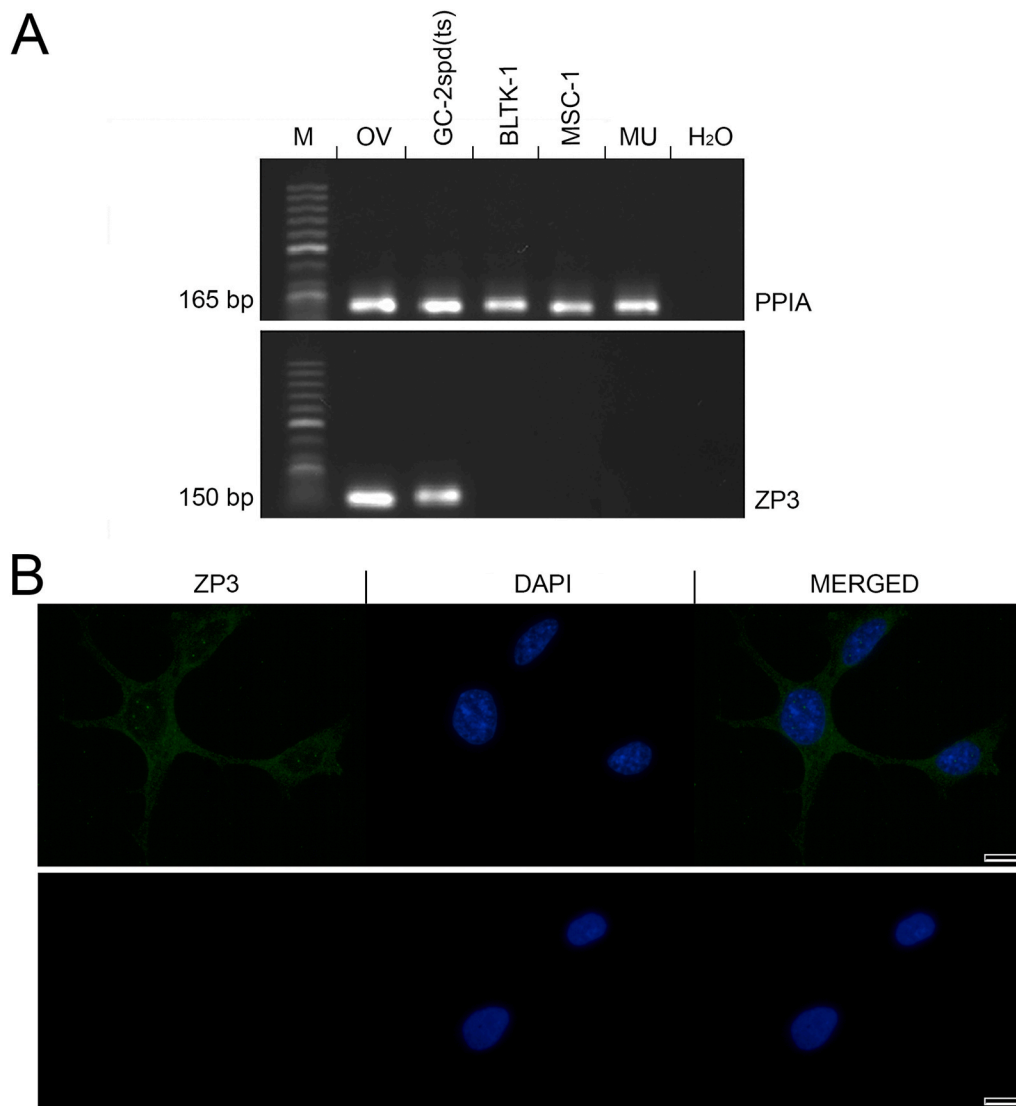
Fig. 4. Zp3 expression in mouse GC-2spd(ts) cell line.
The RT-PCR analysis in 2% agarose gel of housekeeping gene *Ppia* and *Zp3* in GC-2spd(ts) cell line, BLTK-1 cell line, MSC-1 cell line, ovary and muscle (A). Amplicon sizes are presented on the left. Immunocytochemical localization of Zp3 in mouse spermatogenic GC-2spd(ts) cell line (B). The lower panel shows no primary antibody control staining. BLTK-1, murine Leydig cell tumor; GC-2spd(ts), mouse spermatogenic cell line; M, marker; MSC-1, Mouse Sertoli Cell line-1; MU, muscle; OV, ovary; H_2O, nuclease-free water.
Scale bar, 10 μm.

Human Protein Atlas showed ZP3 RNA expression in different normal tissues (in females in the ovary, breast and fallopian tubes and in males in the epididymis, seminal vesicles, prostate, gastrointestinal tract, muscle, brain, adrenal, pituitary, bone and lymphoid tissue) and ZP3 protein expression in the duodenum and small intestine, but no expression in the testis (https://www.proteinatlas.org/ENSG000001 88372-ZP3). For the human protein atlas, they used HPA054061 polyclonal antibody (Sigma-Aldrich), where liver and plasma were negative controls for the ZP3 presence and the U2-OS human osteosarcoma cell line was a positive control, not the ovary, classically expressing the highest amount of ZP3. An osteosarcoma cell line was used as the positive control for ZP3 protein expression, instead of the reference ovarian tissue, which could have led to the inappropriate assignment of ZP3 expression to other tissues. In contrast to the Human Protein Atlas, we did not find protein ZP3 expression in any other healthy normal tissues, except for the ovary and the testis, confirming that results from high-throughput projects should be taken with caution and the need of their verification by independent studies and validation by additional experimental methods (Dammeyer and Arner 2011).

Human protein atlas single-cell RNA sequencing (scRNAseq) data showed male spermatogenic cells express substantially higher levels of ZP3 mRNA than any other cell types for which data were reported, but oocytes were not included in the list (https://www.proteinatlas.org/ENSG00000188372-ZP3). It has been shown that the data obtained from single-cell RNA-sequencing may often give rise to an abundance of negative data, increased variability, and complex expression distributions. Incorrect normalization may cause over-normalization of the weakly/moderately expressed genes (Bacher and Kendziorski 2016). Due to these limitations of the single-cell RNA-sequencing, proper validation of achieved results by other methods (like qPCR, RT-PCR, Western blots, ICC/IHC, *in situ* hybridization etc), as well as by statistical/computational methods are needed. In this paper, we used several techniques to check/confirm the ZP3 expression. We analyzed the ZP3 protein localization with a specific monoclonal antibody and confirmed its presence by RNA transcripts in the testis using a sensitive RNAscope *in situ* hybridization method (Wang et al., 2012).

In female animals, ZP3 immunization induces autoimmune oophoritis and atrophy of the ovaries (Lou et al., 2000). When primordial ovarian follicles are selected for growth and become primary follicles, they start to express ZP antigens in the glycoprotein layer around the oocyte and become susceptible to ZP3-specific antibodies and autoreactive immune cells. It has been demonstrated that antibody binding to ZP3 protein in growing or mature follicles is followed by induction of T cell-mediated inflammation resulting in degeneration of developing follicles (Lou et al., 2000). Recently, it has been shown that in actively immunized mice after careful selection of B-cell epitopes, ZP3 vaccines may induce reversible infertility without adverse effects on the ovaries (Paterson et al., 2002). With a chimeric peptide vaccine, the defined
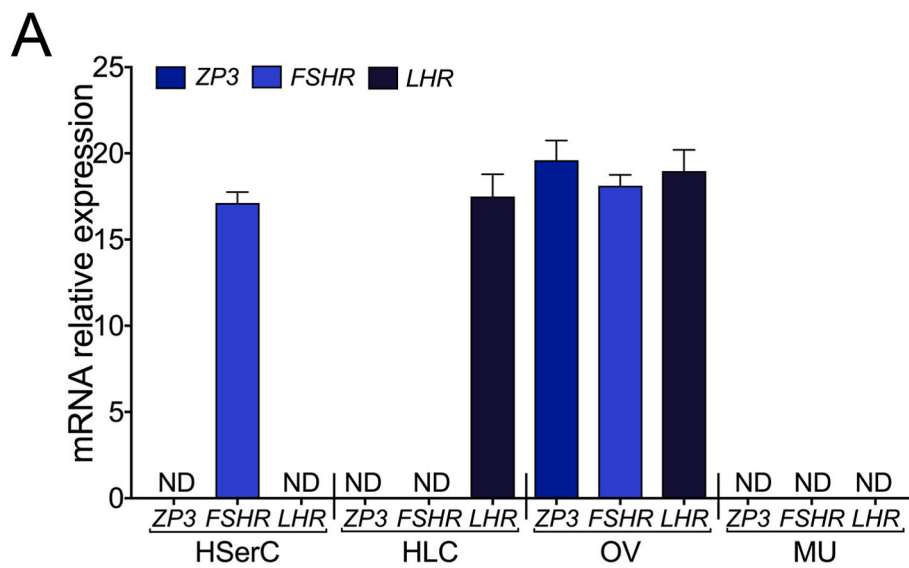
Fig. 5. *ZP3*, *FSHR* and *LHR* expression profiles in human primary Sertoli and Leydig cells. The qPCR analysis of *ZP3, FSHR* and *LHR* expression in human primary HSerC and HLC cells and normal human ovary and muscle (A). The expression of *ZP3* was normalized to that of *PPIA* expression in the same sample. The RT-PCR analysis in 2% agarose gel of *ZP3*, *FSHR*, *LHR* and housekeeping gene *PPIA* in human primary HSerC and HLC cells and normal human ovary and muscle (B). HLC, primary human Leydig cell line; HSerC, primary human Sertoli cell line; M, marker; MU, muscle; OV, ovary; H_2O, nuclease-free water.
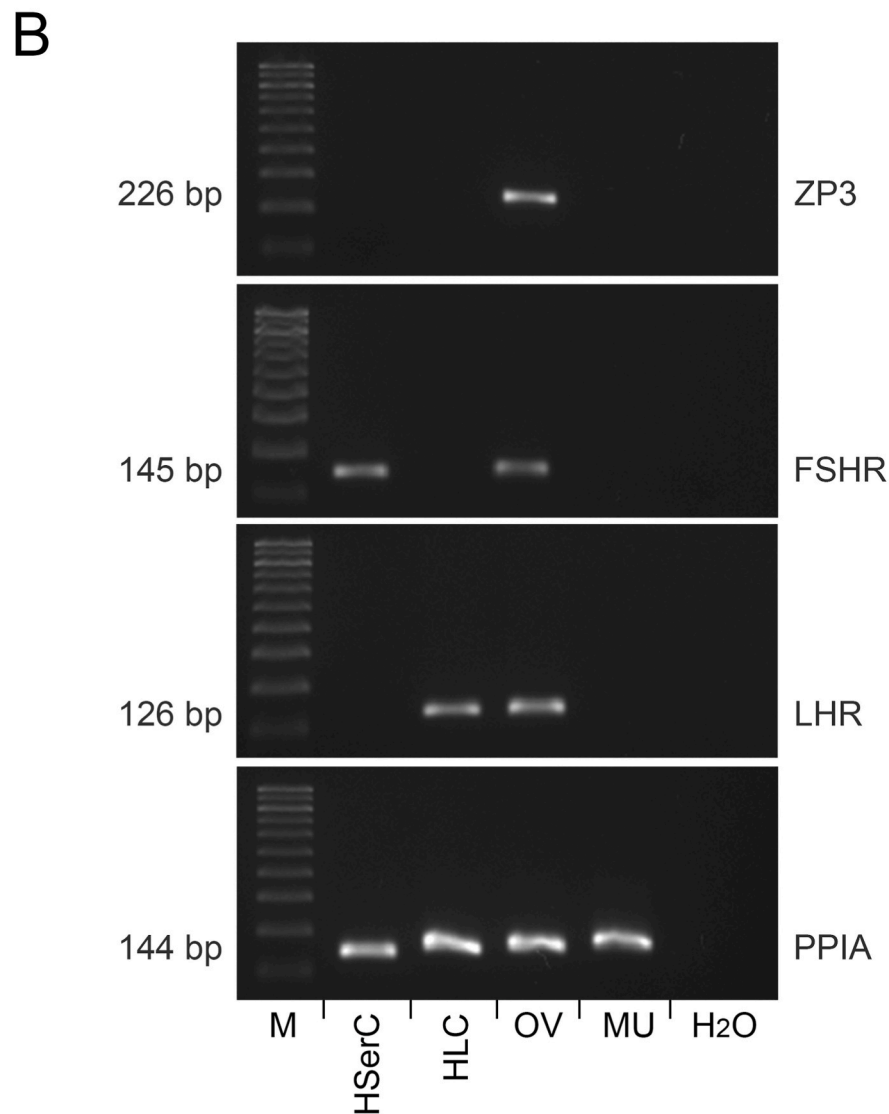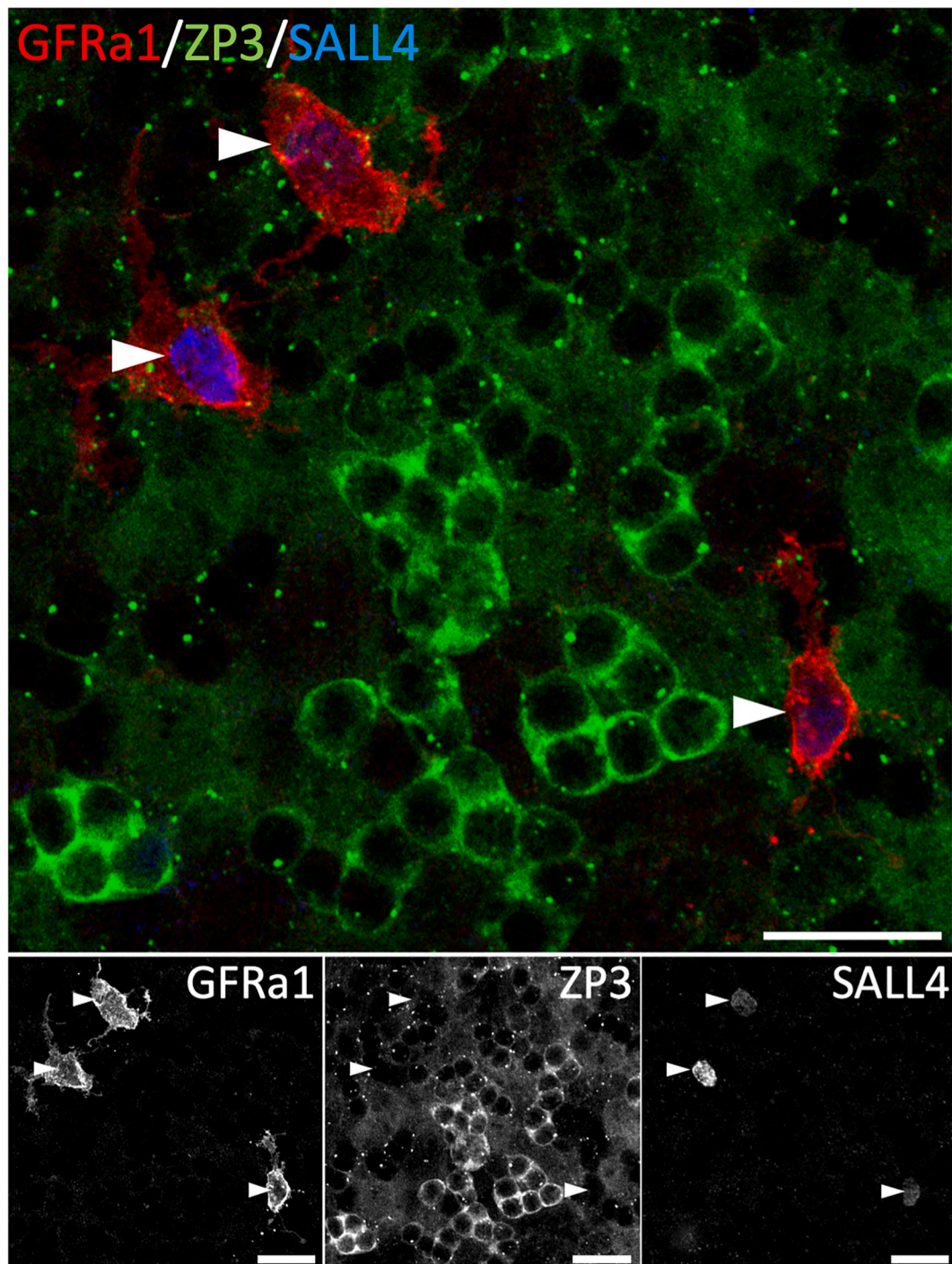
Fig. 6

Fig. 6. Immunohistochemical localization of ZP3 in mouse spermatogonial stem and progenitor cells, SSPCs. A confocal microscopy image of stage VI-VIII adult mouse whole-mount seminiferous tubule staining using antibodies against SSPC marker GFRa1 (red), ZP3 (green) and a pan-spermatogonial marker SALL4 (blue). White arrowheads indicate GFRa1/SALL4-positive A-single (A$_s$) undifferentiated spermatogonia. ZP3 is not expressed in A$_s$ SSPCs. ZP3-positive cells in the middle of the image are preleptotenes spermatocytes (white arrows).
Scale bars 25 μm.

B-cell epitope had a single critical amino acid substitution to prevent cross-reaction with a native ZP3 T-cell epitope, but not B-cell response to ZP3 (Lou et al., 1995). This ZP3 vaccine was able to successfully prevent pregnancy without causing ovarian pathology.

Only one study reported ZP3 expression in human oocytes and granulosa cells as early as the primordial follicle stage (Gook et al., 2008), suggesting ZP protein expression since oogenesis. However, this study used a polyclonal antibody (Harris et al., 1994) not validated by others. In contrast to this study, and in line with the rest of the literature (Bleil and Wassarman 1980; Wassarman and Litscher 2013, Canosa et al., 2017), we did not find any ZP3 expression (either protein or mRNA transcripts) in the primordial follicles or granulosa cells in the present study, using monoclonal ZP3 antibodies. This result confirmed our earlier study with the Zonagen/Repros polyclonal antibody

(Rahman et al., 2012).

Women have a variety of contraceptive methods to choose from, but male contraceptive choices are still limited (Amory 2016). Condoms are the most popular option, but are unreliable (Duerr et al., 2011). A second option is a vasectomy, but this method is invasive and largely irreversible (Kanakis and Goulis 2015). Hormone-based (androgen alone or in combination with a progestin) male contraceptive methods have been tested extensively, but have failed to suppress spermatogenesis in all men completely (Oduwole and Huhtaniemi 2014). These hormonal methods often cause side effects due to the administration of high doses of testosterone and progestin preparations, such as acne, mood changes, weight gain, night sweats and altered libido (Meriggiola et al., 2003). Currently, several projects (such as combined nestorone-testosterone gel or male contraceptive pill using a progestogenic androgen – dimethandrolone 17 beta-undecatone) to develop a hormone-based male contraceptive are ongoing, but adequate efficacy and safety have not been proven yet (Serfaty 2020). There is no doubt that breakthroughs are needed to revive the stagnant field of male contraceptive development.

ZP3 antigen expression in the testis introduces the surprising possibility to use ZP3 immunization for male contraception. The absence of ZP3 expression in any other normal male tissues, as well as in somatic testicular cells, reduces the risk of concomitant damage to other organs. However, the ZP3 epitopes should be carefully selected to avoid the destruction of testicular tissue and allow the ability to cross the blood-testis barrier (Cheng and Mruk 2012). Another important feature of such immunization would be its reversibility. Also, since ZP3 is not expressed by the testosterone synthesizing Leydig cells in the testis, unfavorable endocrine side effects may not occur, provided no general auto-immune response occurs due to adequate selection of the epitopes. Furthermore, since ZP3 expression was not detected in the SSPCs, as well as in Sertoli cells, it is unlikely that anti-ZP3 male contraceptive therapy would destroy spermatogenic stem cells to induce irreversible infertility; hence this male contraceptive strategy could be reversible upon discontinuation.

In conclusion, we report here the novel extraovarian ZP3 expression in human and mouse spermatogenic cells, and the absence of ZP3 expression in a series of normal human and mouse tissues. These findings on absent ZP3 expression in normal nongonadal tissues provide crucial background information for any ZP3 cancer immunotherapy. Any future ZP3 cancer immunotherapy clinical trials in males (such as prostate, lung or colorectal cancer) should proceed with caution due to the ZP3 expression in testis. Besides cancer immunotherapy, another therapeutic strategy was proposed by the finding on ZP3 expression in the testis. The exclusive localization of testicular ZP3 expression in spermatogonia, spermatocytes and spermatids make ZP3 immunization a promising new target for male immunocontraception. Further studies should also address the functional role of ZP3 in the testis, and in particular whether ZP3 immunization has a long-term or reversible effect on male fertility.

Declaration of competing interest

The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study. HJTCB is the owner of the Pandora Endocrine Innovation.

Simple summary

Ovarian oocyte-specific zona pellucida glycoprotein 3 (ZP3) expression was found recently in several cancer tissues, including those of the prostate and lung. The efficacy of ZP3 immunization has been demonstrated in the treatment of ZP3-positive ovarian cancer in transgenic mice, suggesting the efficacy of similar therapy for other ZP3-positive cancers. Hence, we examined whether any other normal tissue besides the ovary may express ZP3, to exclude potential off-target effects of cancer ZP3 immunotherapy. Unexpectedly, ZP3 expression was found in spermatogonia, spermatocytes and round and elongated spermatids of human and mouse testis, but absent in mature spermatozoa, Sertoli, Leydig, spermatogonial stem and progenitor cells, and in an array of 14 different tissues examined. The novel ZP3 expression in testis needs to be taken into consideration during putative ZP3 cancer immunotherapy. ZP3 expression in testicular germ cells, but absence in spermatogenic stem and somatic cells, makes ZP3 a potential target for male immunocontraception.

Declaration of competing interest

HJTCB is the owner of the Pandora Endocrine Innovation. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this study.

CRediT authorship contribution statement

Kamila Pulawska: performed, the experiments, analyzed and interpreted the results, Writing – original draft. **Donata Ponikwicka-Tyszko:** performed the experiments, analyzed and interpreted the results, Writing – original draft. **Weronika Lebiedzinska:** performed the experiments, analyzed and interpreted the results. **Peilan Guo:** performed the experiments. **Piotr Bernaczyk:** performed the experiments. **Agata Pilaszewicz-Puza:** performed the experiments. **Xiangdong Li:** analyzed and interpreted the results. **Marcin Chrusciel:** performed the experiments. **Oana Lupu:** performed the experiments. **Sini Leskinen:** performed the experiments. **Juho-Antti Makela:** performed the experiments. **Jorma Toppari:** analyzed and interpreted the results. **Slawomir Wolczynski:** analyzed and interpreted the results, Writing – original draft. **Herjan J.T. Coelingh Bennink:** analyzed and interpreted the results, Writing – original draft. **Ilpo Huhtaniemi:** analyzed and interpreted the results, Writing – original draft. **Nafis A. Rahman:** analyzed and interpreted the results, Writing – original draft, All authors have approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2021.111502.

References

Amory, J.K., 2016. Male contraception. Fertil. Steril. 106 (6), 1303–1309.

Bacher, R., Kendziorski, C., 2016. Design and computational analysis of single-cell RNA-sequencing experiments. Genome Biol. 17, 63.

Bennink, H.J.T.C., 2016. Immunotherapeutic Method for Treating Prostate Cancer. Pantarhei Bioscience BV, Zeist, Netherlands. US patent 9295720B2.

Bleil, J.D., Wassarman, P.M., 1980. Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture in vitro. Proc. Natl. Acad. Sci. U. S. A. 77 (2), 1029–1033.

Bleil, J.D., Wassarman, P.M., 1983. Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev. Biol. 95 (2), 317–324.

Buschmann, T., Fotiadis, N.-K., Fuchs, M., Heim, S., Lehnherr-Ilina, T., Lamer, S., Meuer, J., Rothmann-Cosic, K., Seibert, V., Tortola Perez, S., Stedronsky, K., Inventor, 2004. Differentially Expressed Tumour-specific Polypeptides for Use in the Diagnosis and Treatment of Cancer, CA Patent CA2539490A1. https://patents.google.com/patent/CA2539490A1/un Genmab AS CA patent CA2539490A1.

Canosa, S., Adriaenssens, T., Coucke, W., Dalmasso, P., Revelli, A., Benedetto, C., Smitz, J., 2017. Zona pellucida gene mRNA expression in human oocytes is related to oocyte maturity, zona inner layer retardance and fertilization competence. Mol. Hum. Reprod. 23 (5), 292–303.

Chan, A.L., La, H.M., Legrand, J.M.D., Makela, J.A., Eichenlaub, M., De Seram, M., Ramialison, M., Hobbs, R.M., 2017. Germline stem cell activity is sustained by SALL4-dependent silencing of distinct tumor suppressor genes. Stem Cell Reports 9 (3), 956–971.

Chen, T., Bian, Y., Liu, X., Zhao, S., Wu, K., Yan, L., Li, M., Yang, Z., Liu, H., Zhao, H., Chen, Z.J., 2017. A recurrent missense mutation in ZP3 causes empty follicle syndrome and female infertility. Am. J. Hum. Genet. 101 (3), 459–465.

Cheng, C.Y., Mruk, D.D., 2012. The blood-testis barrier and its implications for male contraception. Pharmacol. Rev. 64 (1), 16–64.

Coelingh Bennink, H.J.T., Rahman, N.A., Inventor, 2019. Immunotherapeutic Methods for Treating And/or Preventing Lung Cancer. Pantarhei Bioscience BV, Zeist, Netherlands, WO2019086507A1. WO2019086507A1. https://patents.google.com/patent/WO2019086507A1/en.

Costa, J., Pereira, R., Oliveira, J., Alves, A., Marques-Magalhaes, A., Frutuoso, A., Leal, C., Barros, N., Fernandes, R., Queiroz Almeida, D., Barreiro, M., Barros, A., Sousa, M., Sa, R., 2018. Structural and molecular analysis of the cancer prostate cell line PC3: oocyte zona pellucida glycoproteins. Tissue Cell 55, 91–106.

Dammeyer, P., Arner, E.S., 2011. Human Protein Atlas of redox systems - what can be learnt? Biochim. Biophys. Acta 1810 (1), 111–138.

Duerr, A., Gallo, M.F., Warner, L., Jamieson, D.J., Kulczycki, A., Macaluso, M., 2011. Assessing male condom failure and incorrect use. Sex. Transm. Dis. 38 (7), 580–586.

East, I.J., Gulyas, B.J., Dean, J., 1985. Monoclonal antibodies to the murine zona pellucida protein with sperm receptor activity: effects on fertilization and early development. Dev. Biol. 109 (2), 268–273.

Gao, L.L., Zhou, C.X., Zhang, X.L., Liu, P., Jin, Z., Zhu, G.Y., Ma, Y., Li, J., Yang, Z.X., Zhang, D., 2017. ZP3 is required for germinal vesicle breakdown in mouse oocyte meiosis. Sci. Rep. 7, 41272.

Gook, D.A., Edgar, D.H., Borg, J., Martic, M., 2008. Detection of zona pellucida proteins during human folliculogenesis. Hum. Reprod. 23 (2), 394–402.

Gupta, S.K., Jethanandani, P., Afzalpurkar, A., Kaul, R., Santhanam, R., 1997. Prospects of zona pellucida glycoproteins as immunogens for contraceptive vaccine. Hum. Reprod. Update 3 (4), 311–324.

Harris, J.D., Hibler, D.W., Fontenot, G.K., Hsu, K.T., Yurewicz, E.C., Sacco, A.G., 1994. Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. DNA Seq 4 (6), 361–393.

Kanakis, G.A., Goulis, D.G., 2015. Male contraception: a clinically-oriented review. Hormones (Basel) 14 (4), 598–614.

Kananen, K., Markkula, M., el-Hefnawy, T., Zhang, F.P., Paukku, T., Su, J.G., Hsueh, A.J., Huhtaniemi, I., 1996. The mouse inhibin alpha-subunit promoter directs SV40 T-antigen to Leydig cells in transgenic mice. Mol. Cell. Endocrinol. 119 (2), 135–146.

Lefievre, L., Conner, S.J., Salpekar, A., Olufowobi, O., Ashton, P., Pavlovic, B., Lenton, W., Afnan, M., Brewis, I.A., Monk, M., Hughes, D.C., Barratt, C.L., 2004. Four zona pellucida glycoproteins are expressed in the human. Hum. Reprod. 19 (7), 1580–1586.

Liu, C., Litscher, E.S., Mortillo, S., Sakai, Y., Kinloch, R.A., Stewart, C.L., Wassarman, P. M., 1996. Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. Proc. Natl. Acad. Sci. U. S. A. 93 (11), 5431–5436.

Lou, Y., Ang, J., Thai, H., McElveen, F., Tung, K.S., 1995. A zona pellucida 3 peptide vaccine induces antibodies and reversible infertility without ovarian pathology. J. Immunol. 155 (5), 2715–2720.

Lou, Y.H., Park, K.K., Agersborg, S., Alard, P., Tung, K.S., 2000. Retargeting T cell-mediated inflammation: a new perspective on autoantibody action. J. Immunol. 164 (10), 5251–5257.

Mahi-Brown, C.A., Yanagimachi, R., Nelson, M.L., Yanagimachi, H., Palumbo, N., 1988. Ovarian histopathology of bitches immunized with porcine zonae pellucidae. Am. J. Reprod. Immunol. Microbiol. 18 (3), 94–103.

Makela, J.A., Hobbs, R.M., 2019. Molecular regulation of spermatogonial stem cell renewal and differentiation. Reproduction 158 (5), R169–R187.

Makela, J.A., Cisneros-Montalvo, S., Lehtiniemi, T., Olotu, O., La, H.M., Toppari, J., Hobbs, R.M., Parvinen, M., Kotaja, N., 2020. Transillumination-assisted dissection of specific stages of the mouse seminiferous epithelial cycle for downstream immunostaining analyses. JoVE 164.

Meriggiola, M.C., Farley, T.M., Mbizvo, M.T., 2003. A review of androgen-progestin regimens for male contraception. J. Androl. 24 (4), 466–483.

Millar, S.E., Chamow, S.M., Baur, A.W., Oliver, C., Robey, F., Dean, J., 1989. Vaccination with a synthetic zona pellucida peptide produces long-term contraception in female mice. Science 246 (4932), 935–938.

Mohd-Lila, M.A., Yee, L.K., Cen, L.S., Bala, J.A., Balakrishnan, K.N., Allaudin, Z.N., Abdul Rahman, S.O., Hani, H., Abdullah, R., 2019. The application of naked DNA plasmid (DrZP3) and recombinant adenovirus (Ad-rZP3) in rat animal model to determine comparative efficacy of ZP3-Immunocontraceptive vaccines. Microb. Pathog. 134, 103572.

Oduwole, O.O., Huhtaniemi, I.T., 2014. Feasibility of male hormonal contraception: lessons from clinical trials and animal experiments. Curr. Mol. Pharmacol. 7 (2), 109–118.

Paterson, M., Wilson, M.R., Morris, K.D., van Duin, M., Aitken, R.J., 1998. Evaluation of the contraceptive potential of recombinant human ZP3 and human ZP3 peptides in a primate model: their safety and efficacy. Am. J. Reprod. Immunol. 40 (3), 198–209.

Paterson, M., Jennings, Z.A., Wilson, M.R., Aitken, R.J., 2002. The contraceptive potential of ZP3 and ZP3 peptides in a primate model. J. Reprod. Immunol. 53 (1–2), 99–107.

Rahman, N.A., Bennink, H.J., Chrusciel, M., Sharp, V., Zimmerman, Y., Dina, R., Li, X., Ellonen, A., Rivero-Muller, A., Dilworth, S., Ghaem-Maghami, S., Vainio, O., Huhtaniemi, I., 2012. A novel treatment strategy for ovarian cancer based on immunization against zona pellucida protein (ZP) 3. Faseb. J. 26 (1), 324–333.

Rankin, T.L., Tong, Z.B., Castle, P.E., Lee, E., Gore-Langton, R., Nelson, L.M., Dean, J., 1998. Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. Development 125 (13), 2415–2424.

Rebois, R.V., 1982. Establishment of gonadotropin-responsive murine leydig tumor cell line. J. Cell Biol. 94 (1), 70–76.

Serfaty, D., 2020. Why male modern contraception? European Gynecology and Obstetrics 3–4.

Shabanowitz, R.B., 1990. Mouse antibodies to human zona pellucida: evidence that human ZP3 is strongly immunogenic and contains two distinct isomer chains. Biol. Reprod. 43 (2), 260–270.

Wang, F., Flanagan, J., Su, N., Wang, L.C., Bui, S., Nielson, A., Wu, X., Vo, H.T., Ma, X.J., Luo, Y., 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagn. 14 (1), 22–29.

Wassarman, P.M., 1999a. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. Cell 96 (2), 175–183.

Wassarman, P.M., 1999b. The Parkes Lecture. Zona pellucida glycoprotein mZP3: a versatile player during mammalian fertilization. J. Reprod. Fertil. 116 (2), 211–216.

Wassarman, P.M., Litscher, E.S., 2013. Biogenesis of the mouse egg's extracellular coat, the zona pellucida. Curr. Top. Dev. Biol. 102, 243–266.

Wassarman, P.M., Mortillo, S., 1991. Structure of the mouse egg extracellular coat, the zona pellucida. Int. Rev. Cytol. 130, 85–110.

WHO, 2010. Laboratory Manual for the Examination and Processing of Human Sperm, fifth ed. World Health Organization.

Zhou, Z., Ni, C., Wu, L., Chen, B., Xu, Y., Zhang, Z., Mu, J., Li, B., Yan, Z., Fu, J., Wang, W., Zhao, L., Dong, J., Sun, X., Kuang, Y., Sang, Q., Wang, L., 2019. Novel mutations in ZP1, ZP2, and ZP3 cause female infertility due to abnormal zona pellucida formation. Hum. Genet. 138 (4), 327–337.