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Cite this article: Babatabar Darzi M *et al.* (2023) Immunohistochemistry and immunocytochemistry analysis of PLZF and VASA in mice testis during spermatogenesis. *Zygote*. **31**: 273–280. doi: 10.1017/S0967199423000047

Received: 5 August 2022
Revised: 9 December 2022
Accepted: 24 January 2023
First published online: 3 April 2023

Keywords:
Germ cells; PLZF; Spermatogonial stem cells; VASA

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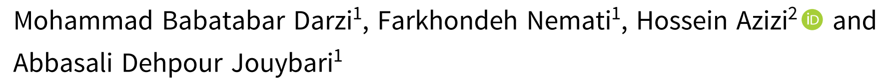
Immunohistochemistry and immunocytochemistry analysis of PLZF and VASA in mice testis during spermatogenesis



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Summary

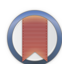
Spermatogonial stem cells (SSCs) are the basis of male spermatogenesis and fertility. SSCs are distinguished by their ability to self-renew and differentiate into spermatozoa throughout the male reproductive life and pass genetic information to the next generation. Immunohistochemistry (IHC), immunocytochemistry (ICC) and Fluidigm reverse transcriptase-polymerase chain reaction (RT-PCR) were used to analyze the expression of PLZF and VASA in mice testis tissue. In this experimental study, whereas undifferentiated spermatogonial cells sharply expressed PLZF, other types of germ cells located in the seminiferous tubule were negative for this marker. Conversely, the germ cells near the basal membrane of the seminiferous tubule showed VASA expression, whereas the undifferentiated germ cells located on the basal membrane were negative. The ICC analysis indicated higher expression of PLZF in the isolated undifferentiated cells compared with differentiated germ cells. Fluidigm real-time RT-PCR results demonstrated a significant expression ($P < 0.05$) of VASA in the SSCs compared with differentiated cells and also showed expression of PLZF in undifferentiated spermatogonia. These results clearly proved the role of PLZF as a specific marker for SSCs, and can be beneficial for advanced research on *in vitro* differentiation of SSCs to functional sperms.

Introduction

The mammalian testis consists of a complex multicellular system that is divided into two compartments: the seminiferous tubules and the interstitial tissue. Two existing types of cells in the testicular tissue, germ cells (undifferentiated and differentiated cells) and somatic cell (including Sertoli cells, Leydig cells and peritubular myoid cells) carry out the male reproduction tasks, that is transmission of genetic information to the subsequent generation (Azizi *et al.*, 2021a). Spermatogonia cells [also known as spermatogonial stem cells (SSC)], which are localized along the basement membrane of the seminiferous tubules, initiate one of the most important biological process during the male lifetime, spermatogenesis, which finally results in sperm-cell production (Azizi *et al.*, 2021b). As the *in vitro* study of different model systems in reproduction biology may not be fully possible and convenient, organ culture allows modelling of testicular conditions *in vitro*, making it a powerful tool with which to study tissue-specific cell–cell interactions and may provide a platform with which to study biological process precisely, including spermatogenesis. All spermatogenesis stages are controlled by the stem cell factor (SCF) signalling pathway (Zheng *et al.*, 2020).

In mammals, the testis is composed of complex networks of tubes that are unique and responsible for the expression of male reproductive potential. Germ cells and somatic cells collaborate in the testis (Kanbar *et al.*, 2021). In addition, germ cells are responsible for the production of spermatids and then sperm during spermatogenesis (Rezaei Topraggaleh *et al.*, 2019). First, spermatogenesis is initiated by the main germ cells, known as spermatogonia (Spg), which are located on the base membrane of seminiferous tubules. Spgs have two functions after the division, the first function is to renew the main germ cells to retain the pool of progenitor cells and the second function is to produce primary and secondary spermatocytes (Azizi *et al.*, 2020b; Niazi Tabar *et al.*, 2022b). During the final division in spermatogenesis, secondary spermatocytes become spermatids, which differentiate into sperm as male fertility cells. Normal spermatogenesis needs not only normal germ cells but also an appropriate environment in which to provide sufficient nutrition and other chemical factors (Azizi *et al.*, 2020a; Niazi Tabar *et al.*, 2022a).

The *VASA* gene was first found to be essential for the development of female germ stem cells (GSCs) in *Drosophila* (Khadivi *et al.*, 2020). In mice with systematic genetic deletions of the *VASA* gene, males exhibit a reproductive deficiency with a loss of sperm production. The male GSCs die during the zygotene step in meiosis, whereas the ovarian function appears to be normal



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(Abofoul-Azab et al., 2019). It has been observed that VASA is localized in PGCs in mice from embryonic day 12.5 onwards, directly after entering the gonadal anlage (Kanatsu-Shinohara et al., 2022). Previous studies have demonstrated the essential role of PLZF as another marker in the direct repression of the transcription of Kit, a marker of spermatogonia differentiation (Moraveji et al., 2019; Zheng et al., 2020). It has also been demonstrated that loss of the encoding the *PLZF* gene results in limited numbers of normal spermatozoa, which leads progressively to a lack of the respective germline after birth (Kanbar et al., 2021). During embryogenesis, PLZF regulates the gene expression stage for limb and axial skeletal patterning. In the present study we analyzed the co-expression of PLZF and Oct4 in two types of cell populations present in seminiferous tubules (Rahmani et al., 2019).

Infertility in humans is often caused by defective spermatogenesis. For the development of human subfertility and infertility, understanding normal spermatogenesis is essential. Some RNA-binding proteins are needed for germ cell formation. VASA expression in germ cells has been seen in rhesus macaques, goats, cattle, pigs, and other animals. An RNA-binding protein and an ATP-dependent RNA helicase are both encoded by the *VASA* gene. VASA protein expression may be utilized to identify spermatogonia, spermatocytes, and spherical spermatids in human testicular tissues. Understanding the expression patterns of these proteins in diverse germ cells at different stages might aid in the comprehension of human spermatogenesis (Amirian et al., 2022).

Several studies have demonstrated that the Vasa protein, which functions as an RNA chaperone and is connected to the chromatoid body, is dispersed evenly throughout the cytoplasm of *Drosophila* cells. According to different research, when the genome is inactive, VASA acts as the CB and transcribes mRNA that is still present in spermatozoa. VASA is necessary for the differentiation of embryonic stem cells into primordial germ cells and spermatogonium stem cells, in addition to spermatogenesis (Shukalyuk et al., 2007; Gustafson and Wessel, 2010; Amirian et al., 2022).

PLZF may play an important role in SSC development; however, it is uncertain if the PLZF intermediate filament is required during differentiation *in vitro*, and one study on stage association in the rat seminiferous epithelium has been undertaken (Onohara et al., 2010). Finally, there have been few investigations on the expression of PLZF in male germ cells. In this investigation, we looked at the expression of PLZF in seminiferous tubules and germ cells *in vivo* and *in vitro*.

Materials and methods

For the present study, animal experiments were approved by the Ethical Committee of Amol University of Special Modern Technologies (Ir.ausmt.rec.1400.05). C57BL/6 mice used in this study were purchased from the Institute for Anatomy and Cell Biology at the University of Heidelberg (Heidelberg, Germany).

Isolation of spermatogonial stem cells

Testes from 6-day-old mice were collected, decapsulated and digested in an enzyme digestion solution that contained DNase (0.5 mg/ml) (Sigma-Aldrich), dispase (0.5 mg/ml), and collagenase IV (0.5 mg/ml) (Sigma-Aldrich) in Hank's balanced salt solution (HBSS) buffer (PAA, USA).

Characterization of testicular cells

After enzyme digestion, mice testicular cells were fixed using 4% paraformaldehyde, placed on slides and underwent Cytospin™ centrifugation. The slides were washed with phosphate-buffered saline (PBS), blocked with 1% bovine serum albumin (BSA)/PBS, and incubated overnight with anti-PLZF to label spermatogonia and anti-OCT4. The slides were then incubated overnight with fluorochrome species-specific secondary antibodies. The nuclei were stained with 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), and the cells were analyzed using confocal laser scanning fluorescence microscopy.

Testicular culture on STO feeder layer

SSCs were grown on a feeder layer that was generated from a SIM mouse embryo and was thioguanine and ouabain resistant (STO). The culture medium consisted of 1% L-glutamine (PAA, USA), 1% N_2-supplement (Invitrogen, USA), StemPro-34 medium, 5 μg/ml BSA (Sigma-Aldrich, USA), 6 mg/ml D+-glucose (Sigma-Aldrich, USA), 1% penicillin/streptomycin (PAA, USA), 30 ng/ml estradiol (Sigma-Aldrich, USA), 1% non-essential amino acids (PAA, USA), 0,1% β-mercaptoethanol (Invitrogen, USA), 10 ng/ml FGF (Sigma-Aldrich, USA), 60 ng/ml progesterone (Sigma-Aldrich, USA), 100 U/ml human LIF (Millipore), 8 ng/ml GDNF (Sigma-Aldrich, USA), 1% MEM vitamins (PAA, USA), 30 μg/ml pyruvic acid (Sigma-Aldrich, USA), 20 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, USA), 1% ES cell qualified FBS, 1 μl/ml DL-lactic acid (Sigma-Aldrich, USA) and 100 μg/ml ascorbic acid (Sigma-Aldrich, USA) at 37°C and 5% CO_2 in air. This method followed a one-step enzymatic digestion protocol (Azizi et al., 2022; Hashemi Karoii and Azizi, 2022; Hashemi Karoii et al., 2022; Karoii et al., 2022).

Immunohistofluorescence staining

Testicular tissue were picked up after decapsulation of the tunica albuginea, washed with PBS, and fixed in 4% paraformaldehyde. Tissue was dehydrated during tissue processing and surrounded in Paraplast Plus. Then, the tissue was cut with a microtome (usually ~8–10 μm thickness). Sections from testis tissue were mounted on Hydrophilic Plus slides and stored at room temperature until use. During the immunohistofluorescence staining process, slides were washed in xylene and water was slowly replaced through a series of increasing concentrations of ethanol. Before staining, antigen retrieval was carried out by heat-induced epitope retrieval (HIER) methods at 95°C for 20 min and the non-specific binding site in the tissue sections was blocked with 10% serum/0.3% Triton X-100 in PBS. The characterization of immunohistofluorescence and immunocytofluorescence staining for these sections was followed as described in a previous study (Azizi et al., 2016).

Immunocytofluorescence staining

Cells isolated from the testis were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS, blocked with 1% BSA/PBS, and incubated with primary antibodies to PLZF and VASA. The process was continued with an overnight incubation (usually ~16 h) of fluorochrome species-specific secondary antibody at 4°C. The labelled cells were identified by simple nuclear counterstain with 0.2 μg/ml DAPI dye. Antibody-labelled positive cells were examined using a Zeiss LSM 700 confocal laser scanning microscope, and images of cells were obtained using a Zeiss LSM-TPMT camera.

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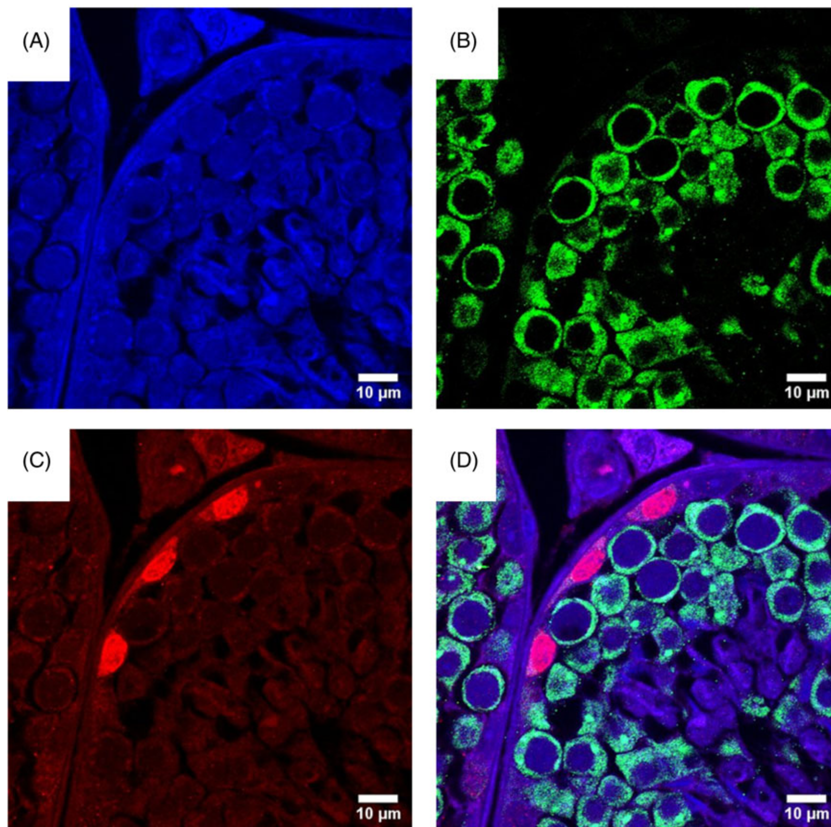

Figure 1. Immunohistofluorescence analysis in testis section. DAPI (A), immunohistofluorescence analysis for expression of PLZF in the testis (B) and VASA (C). Merged image with blue DAPI (D); (scale bars: 10 μm).

Cell viability

The testicular cells were planted onto culture plates (100 mm), cultured for 4 days, and trypsinized for viability determination. Trypan blue solution was added on day 4, and cells incubated for 5 min at room temperature to test the proliferative effect of the growth factor. Finally, the ratio of viable/dead cells was counted and determined.

Fluidigm BioMark system

To determine the level of expression of the *CD117* gene, SSCs and trophoblast stem cells (TSC) cells were examined using the Fluidigm BioMark system. SSCs and TSC cells were picked up using a micromanipulator, lysed in a solution of lysis buffer containing 9 μl RT-PreAmp Master Mix [5.0 μl Cells Direct 2× Reaction Mix (Invitrogen, USA), 0.2 μl RT/*Taq* Superscript III (Invitrogen, USA), 2.5 μl 0.2× assay pool and 1.3 μl Tris-EDTA (TE) buffer]. We then examined the amount of amplified product of RNA-targeted copies with TaqMan Fluidigm real-time PCR on the Fluidigm BioMark system. Samples were analyzed in two technical repeats. The C_t values were calculated using GenEx software and MS Excel.

Search strategy and data preparation for network analysis

Spermatogenesis-related datasets were explored from the gene database (https://www. ncbi.nlm.nih.gov/gene/). The search strategy was (spermatogenesis) AND "Mus musculus" [porgn: _txid10090]. Then, the gene expression profiles were collected in an Excel file. A *P*-value < 0.05 was considered for the selection of gene interactions and clusters.

Protein–protein interactions (PPI) network analysis

The Retrieval of Interacting Genes (STRING v.11) online tool was applied to predict protein–protein biological and functional interactions (https://stringdb.org/) (Szklarczyk *et al.*, 2021). The spermatogenesis genes with a significant role in vimentin were uploaded in the STRING tool. Predicted PPIs were highlighted to identify the master regulator of vimentin and the spermatogenesis-related signalling pathway. The highlighted genes were imported to Cytoscape (version 3.8.2) with the CentiScape plugin for further analysis and PPI network visualization. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Reactome enrichment pathway were investigated using Enrichr, an online software tool for functional gene annotation (http://amp.pharm.mssm.edu/Enrichr/).

Statistical analysis

Expression of PLZF and VASA in two populations of differentiated and undifferentiated cells was analyzed using the independent sample *t*-test. Statistical analysis was performed using IBM SPSS Statistics for Windows, v.25.0 (IBM Corp., Armonk, NY, USA) ($P < 0.05$).

Results

PLZF expression in seminiferous tubules by immunohistochemistry

In the first step, we examined the expression of PLZF and VASA in adult testis through immunohistochemistry (Figure 1). Immunohistochemistry with confocal microscopy revealed that the PLZF protein was expressed in the spermatogonial cells that were localized on the basal membrane of seminiferous tubules

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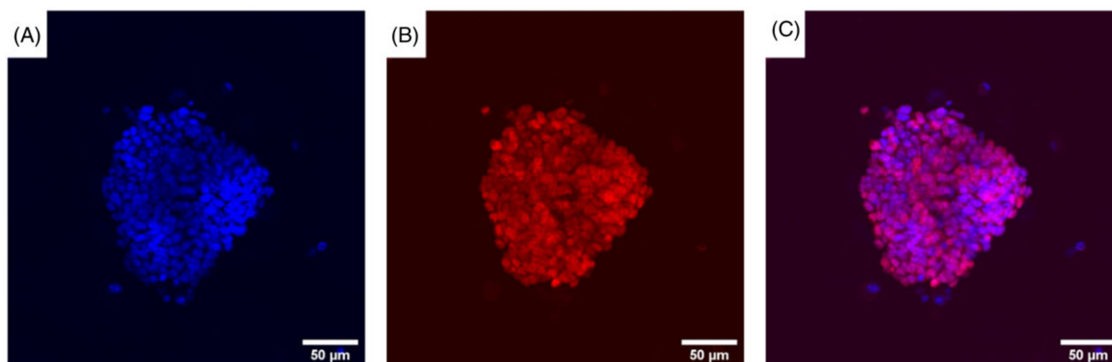

Figure 2. Immunocytofluorescence analysis of PLZF in spermatogonial stem cells. DAPI (A), immunocytofluorescence analysis showing final colonies from the expansion of our extracted spermatogonial stem cells, and expressing PLZF (B). Merged with blue DAPI (C); (scale bars: 50 μm).

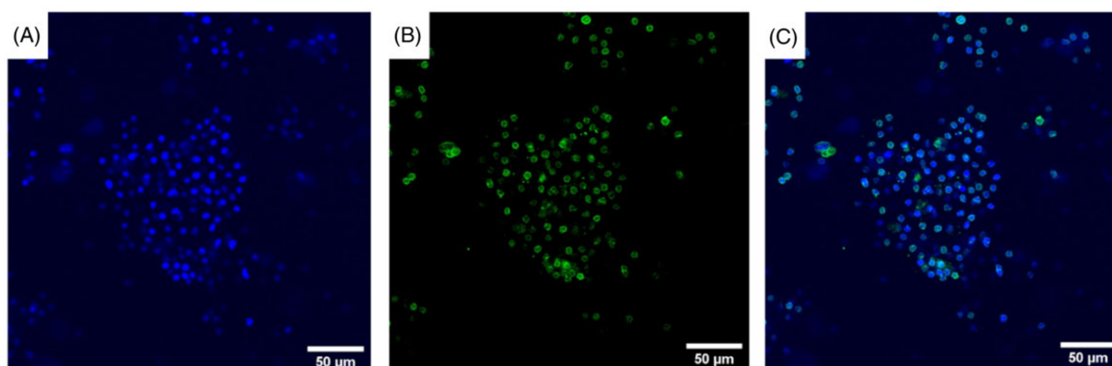

Figure 3. Immunocytofluorescence analysis of PLZF in differentiating spermatogonia. DAPI (A), immunocytofluorescence analysis showing low expression of PLZF in differentiating germ cells (B). Merged with blue DAPI (C); (scale bars: 50 μm).

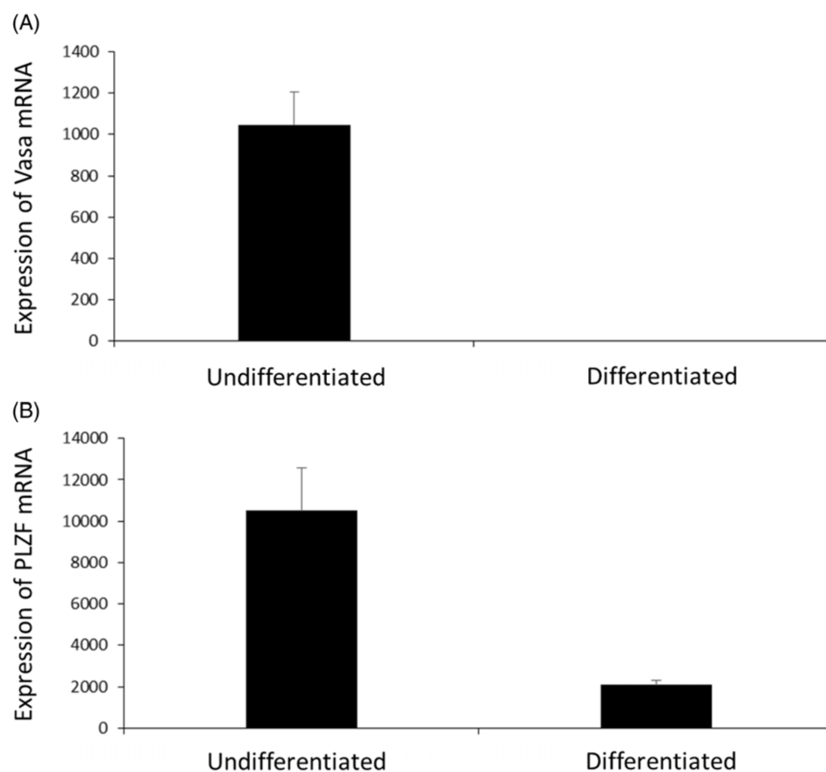

Figure 4. mRNA expression of Vasa and PLZF. Fluidigm real-time PCR analysis of Vasa expression in two populations of differentiated and undifferentiated cells (A). Analysis of PLZF expression in two populations of differentiated and undifferentiated cells (B).

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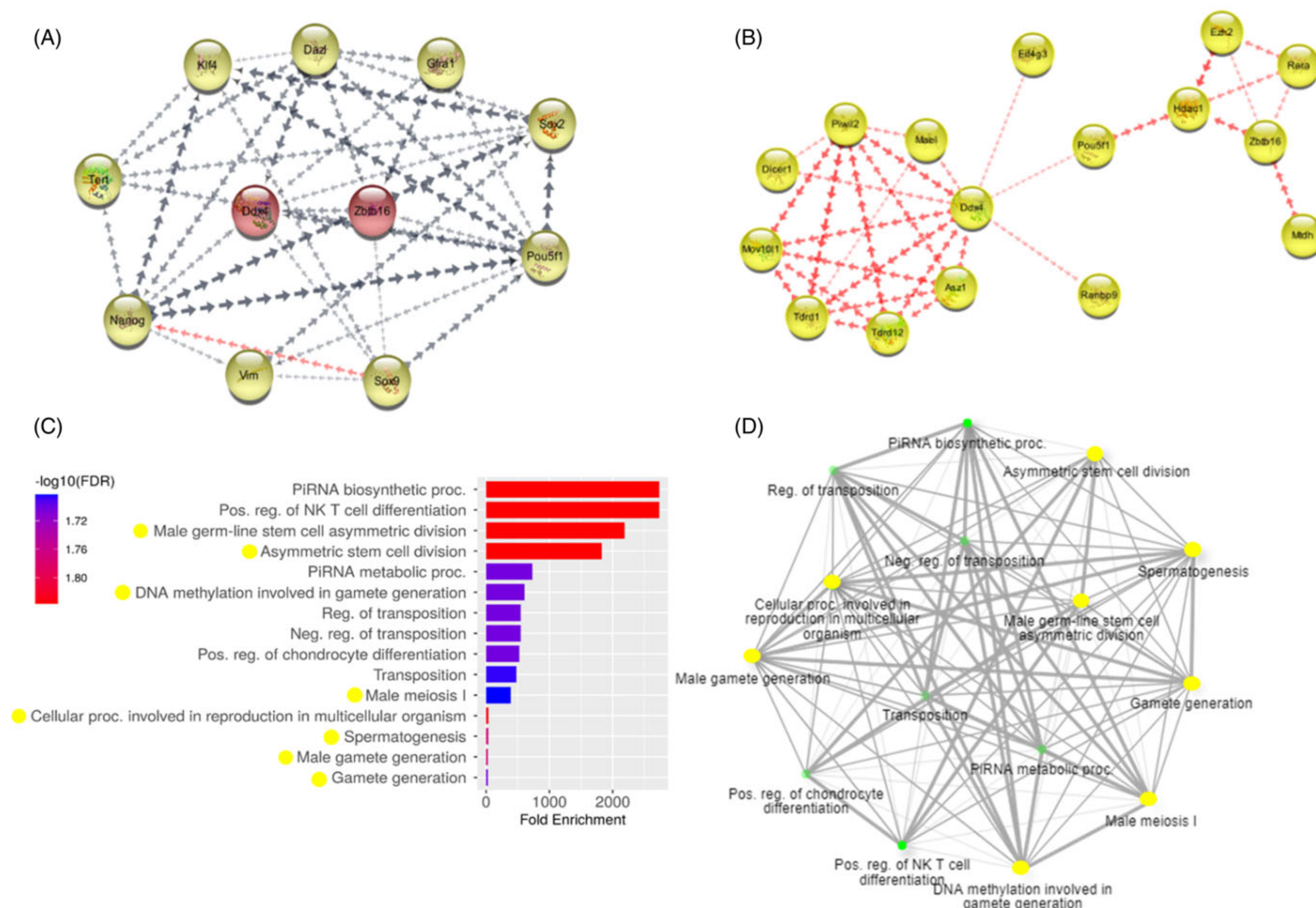

Figure 5. PPI visualization of PLZF and VASA functions in spermatogenesis. The PPI network was visualized using 945 genes from the STRING database. (A) Protein–protein interaction PLZF with spermatogenesis genes. (B) Protein–protein interaction VASA with spermatogenesis genes. (C) Gene ontology of DAZL and VASA involved in spermatogenesis. (D) Gene ontology of DAZL and VASA networks.

(Figure 1A,B). In the adult testis sections, VASA-positive cells were distributed throughout the spermatogonia, spermatocytes, and spermatids with the exclusion of SSCs located in the cell layer directly connected to the base membrane of the seminiferous tubule and were also abundant in sperm (Figure 1C).

Testicular cells isolation and vimentin's expression by immunocytochemical analysis

As the next step, by immunocytochemistry we evaluated the expression level of PLZF in adult SSCs after isolation and culture on a feeder layer. ICC images revealed that the generated SSCs were positive for PLZF, similar to findings under *in vivo* conditions (Figure 2), whereas the expression level of this marker was extremely low in differentiating germ cells (Figure 3).

Our real-time PCR analysis as a support for our immunostaining revealed that the expression of PLZF mRNA in SSCs was significantly higher than in other germ cells ($P < 0.05$) in contrast with VASA mRNA that showed no significant difference in undifferentiated and differentiated spermatogonia (Figure 4).

Protein–protein interaction visualization of PLZF in spermatogenesis

The protein–protein interaction network was visualized with 650 genes using the STRING (v.11) database. It demonstrated that there was a close relationship between interaction and regulated PLZF in the spermatogenesis process. We observed a high level of interaction between Tert, Nanog, vimentin, Sox2, Sox9, Gfra1, Zbtb16, POU5f1, Klf4, DAZL, DDX4 and PLZF. In addition, there was a clear association among Tert, Nanog, and vimentin. Reactome (https://reactome.org/) and KEGG (https://www.genome.jp/kegg/) selected any spermatogenesis-related signalling pathway to highlight the master regulator of the spermatogenesis pathways. There was a strong correlation between the highlighted genes, as shown in Figure 5.

Functional enrichments in the PPI network

To identify the enriched biological processes and molecular activities connected to VASA (DDX4), enrichment analysis was conducted (Figure 5C). Control of the reproductive process, piRNA binding, fertilization, male meiosis I, cell cycle, RNA binding, and other tasks exhibiting hub genes, was among the biological processes we chose depending on the goals of our investigation (Figure 5D).

Discussion

Spermatogonia cells (also known as spermatogonial stem cells, SSC), which are localized along the basement membrane of seminiferous tubules, initiate one of the most important biological

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Table 1. Close relationship protein–protein interaction between differentiation and regulation in spermatogenesis

Related gene	Description	References
KLF4	Klf4 has been identified as a transcription factor required for epithelial cell post-proliferative differentiation. We demonstrate that Klf4 is expressed strongly in post-meiotic germ cells undergoing final differentiation into sperm cells and that it is also expressed in somatic Sertoli cells. These data show that Klf4 may play a crucial role in mammalian testicular differentiation	Shi and Ai, 2013
Vim	Proving that vimentin is an intermediate filament with crucial roles in the differentiation stages of testicular germ cells. Vimentin is connected to the mitochondria and endoplasmic reticulum either laterally or terminally	Hashemi Karoii and Azizi, 2022; Karoii *et al.*, 2022; Niazi Tabar *et al.*, 2022a
POU5F1	During normal development, POU5F1 controls pluripotency. Pou5f1/POU5F1 plays an important role in differentiation by regulating cells with pluripotent capacity. According to the findings, POU5F1 downregulation in differentiating spermatogonia is an important phase in the spermatogenesis process	Niazi Tabar *et al.*, 2022a
Ddx4	DEAD-box polypeptide-4 (Ddx4) is involved in embryogenesis, spermatogenesis, and cellular growth and division	Guan *et al.*, 2017
DAZL	The deleted in azoospermia-like (*DAZL*) gene is involved in spermatogenesis through controlling the formation of spermatids in post-pubertal rams, as well as a unique involvement in functional spermatogonia maintenance	Li *et al.*, 2019
SOX2	SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development	Adikusuma *et al.*, 2017
SOX9	Sox9 is involved in Sertoli cell differentiation, the activation of Mis and Sox8, and the inactivation of Sry	Barrionuevo *et al.*, 2009
Zbtb16	The differential activation of the Zbtb16 and c-Kit genes in neighbouring spermatogonia germ cells was caused by the selective activation of classical or nonclassical signalling pathways in Sertoli cells inside testis explants. The delivery of an inhibitor of either route to mouse testicular Sertoli cells damaged the blood–testis barrier, which is required for spermatogenesis	Marcon *et al.*, 2011
Nanog	During the mitotic arrest, the number of NANOG-positive germ cells dramatically decreased. Adult mouse testes and ovaries had no NANOG-positive germ cells. NANOG is expressed in proliferating germ cells during germ cell development	Yamaguchi *et al.*, 2005
GFRA1	GDNF family receptor alpha 1 (GFRA1) plays a pivotal role in maintaining spermatogonial stem cells in an undifferentiated state	Grasso *et al.*, 2012
Tert	Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by the addition of the telomere repeat TTAGGG, expressed during testicular differentiation in mammals	Bian *et al.*, 2006; Turnbull *et al.*, 2010

process during male lifetimes, spermatogenesis, which finally results in sperm-cell production (Wei *et al.*, 2021). Immunohistochemistry analysis indicated the presence of PLZF-positive cells on the basal membrane of seminiferous tubules. It seems that the PLZF germ cell marker was expressed specifically in spermatogonial cells in the testis. Confocal microscopy characterization for the testis section demonstrated the localization of VASA-positive cells near the basal compartment.

The interaction and regulation of PLZF in the spermatogenesis process were shown to be closely related. Tert, Nanog, vimentin, Sox2, Sox9, Gfra1, Zbtb16, POU5f1, Klf4, DAZL, and DDX4 are all important regulators of chromosomal segregation in meiosis. Vimentin is the key regulator of meiotic spermatogenesis pathways. Therefore, PLFZ coregulation may involve the meiotic phase.

In the spermatogenesis process, there was a tight association between interaction and regulated PLFF. Tert, Nanog, vimentin, and PLZF interacted extensively. To highlight the master regulator of the spermatogenesis pathways, Reactome and KEGG chose any spermatogenesis-related signalling pathway. There was a strong link between the highlighted genes.

We used datasets relating to protein–protein interaction members in this investigation because of a paucity of information about PLZF expression at various spermatogenic stages. The gene expression ontology study revealed that multiple biological and functional pathways were involved in PLZF expression at different stages of spermatogenesis. This protein's increasing expression, however, is linked to cell differentiation. Recently, PLZF expression has been linked to SSC differentiation, localization, and signalling pathways through cell surface receptors. Biological and functional investigation revealed that the *Stat3*, *Mmp2*, *Trp53*, *Casp7*, *AURKB*, *Pik3r1*, *Ctnnb1*, *Lgals3*, *Cdkn1a*, *Snai1*, and *Pou5f1* genes increased PLZF expression (Table 1). The table describes genetic and biological roles in germ cell differentiation. There was a close relationship between interaction and regulated vimentin in the spermatogenesis process. There was high interaction among Stat3, Mmp2, Trp53, Casp7, AURKB, Pik3r1, Ctnnb1, Lgals3, Cdkn1a, Snai1, and Pou5f1. In addition, there was a clear association between Trp53, Mmp2, Casp7, Stat3, and Pik3r1. Reactome and KEGG selected any spermatogenesis-related signalling pathway to highlight the master regulator of the spermatogenesis pathways. There was a powerful correlation among the highlighted genes.

In the present experiment after SSCs generation under stimulation with growth factors FGF, EGF and GDNF, our immunocytochemistry staining demonstrated the sharp expression of PLZF and VASA in SSCs in contrast with *in vivo* conditions in which VASA had a low expression in these cells. Data obtained from IHC analysis indicated that VASA was expressed in the centre of the testicular cords. We observed the expression of the VASA protein in spermatocytes located above the spermatogonial cell layer in the seminiferous tubule of the adult mouse testis, and a decrease in VASA protein expression during spermiogenesis. This might be due to histological changes in this compartment, including separation from Sertoli cells and the feeder cells. In fact, germ cell fate requires key gene regulation, hormones regulators and other chemical and physical support that remains poorly understood for 2D and 3D

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culture medium conditions (Kang *et al.*, 2020; Zheng *et al.*, 2020). Two-dimensional culture has played an important role for reproductive biology studies (Richer *et al.*, 2020). As in other 2D cultural systems, co-culture systems of germ cells and somatic cells allowed insights into how these cells and extracellular matrix proteins in testis remain together in close contact (Sakib *et al.*, 2020). Recently, studies used three-dimensional culture or organoid culture that made their results more translatable to the situation *in vivo* (Alves-Lopes and Stukenborg, 2018; Sakib *et al.*, 2019).

Our results agreed with those of previous studies, suggesting that PLZF is a specific marker for SSCs both *in vivo* and *in vitro* and VASA is a germline marker during spermatogenesis and also in proliferating spermatogonia that are expressed more specifically in SSCs under *in vitro* conditions (Strange *et al.*, 2018).

These findings not only conclusively demonstrated the importance of PLZF as a particular marker for SSCs, but they also suggested that further research into the *in vitro* differentiation of SSCs into functional sperm may benefit from these findings.

Acknowledgements. The present article was extracted from a PhD dissertation from the Animal Biology (Cellular Developmental) at the Department of Biology, Qaemshahr Branch, Islamic Azad University, Qaemshahr, Iran in 2022. I first should express my gratitude to my supervisor Farkhondeh Nemati. This research was supported by the Faculty of Biotechnology of Amol University of Special Modern Technologies.

Author contributions. Mohammad Babatabar Darzi: designed and carried out experiments, and assembled and analyzed data. Hossein Azizi: designed and carried out experiments, and edited the manuscript. Farkhondeh Nemati: provided critical feedback, analyzed data, and edited the manuscript. All authors read and approved the final manuscript.

Funding. This research received no external funding.

References

Abofoul-Azab, M., Lunenfeld, E., Levitas, E., Zeadna, A., Younis, J. S., Bar-Ami, S. and Huleihel, M. (2019). Identification of premeiotic, meiotic, and postmeiotic cells in testicular biopsies without sperm from Sertoli cell-only syndrome patients. *International Journal of Molecular Sciences*, **20**(3), 470. doi: 10.3390/ijms20030470

Adikusuma, F., Pederick, D., McAninch, D., Hughes, J. and Thomas, P. (2017). Functional equivalence of the SOX2 and SOX3 transcription factors in the developing mouse brain and testes. *Genetics*, **206**(3), 1495–1503. doi: 10.1534/genetics.117.202549

Alves-Lopes, J. P. and Stukenborg, J. B. (2018). Testicular organoids: A new model to study the testicular microenvironment *in vitro*? *Human Reproduction Update*, **24**(2), 176–191. doi: 10.1093/humupd/dmx036

Amirian, M., Azizi, H., Hashemi Karoii, D. and Skutella, T. (2022). VASA protein and gene expression analysis of human non-obstructive azoospermia and normal by immunohistochemistry, immunocytochemistry, and bioinformatics analysis. *Scientific Reports*, **12**(1), 17259. doi: 10.1038/s41598-022-22137-9

Azizi, H., Conrad, S., Hinz, U., Asgari, B., Nanus, D., Peterziel, H., Hajizadeh Moghaddam, A., Baharvand, H. and Skutella, T. (2016). Derivation of pluripotent cells from mouse SSCs seems to be age dependent. *Stem Cells International*, **2016**, 8216312. doi: 10.1155/2016/8216312

Azizi, H., Niazi Tabar, A. and Mohammadi, A. (2020a). Experimental investigation of Ki67, POU5F1, and ZBTB16 expression in the pig and mouse testicular cells using immunocytochemistry and RT-PCR. *Journal of Īlām University of Medical Sciences*, **28**(5), 1–10. doi: 10.29252/sjimu.28.5.1

Azizi, H., Niazi Tabar, A. N., Skutella, T. and Govahi, M. (2020b). *In vitro* and *in vivo* determinations of the anti-GDNF family receptor alpha 1 antibody in mice by immunochemistry and RT-PCR. *International Journal of Fertility and Sterility*, **14**(3), 228–233. doi: 10.22074/ijfs.2020.6051

Azizi, H., Niazi Tabar, A. and Skutella, T. (2021a). Successful transplantation of spermatogonial stem cells into the seminiferous tubules of busulfan-treated mice. *Reproductive Health*, **18**(1), 189. doi: 10.1186/s12978-021-01242-4

Azizi, H., Niazi Tabar, A., Mohammadi, A. and Skutella, T. (2021b). Characterization of *DDX4* gene expression in human cases with non-obstructive azoospermia and in sterile and fertile mice. *Journal of Reproduction and Infertility*, **22**(2), 85–91. doi: 10.18502/jri.v22i2.5793

Azizi, H., Hashemi Karoii, D. and Skutella, T. (2022). Whole exome sequencing and in silico analysis of human Sertoli in patients with non-obstructive azoospermia. *International Journal of Molecular Sciences*, **23**(20), 12570. doi: 10.3390/ijms232012570

Barrionuevo, F., Georg, I., Scherthan, H., Lécureuil, C., Guillou, F., Wegner, M. and Scherer, G. (2009). Testis cord differentiation after the sex determination stage is independent of Sox9 but fails in the combined absence of Sox9 and Sox8. *Developmental Biology*, **327**(2), 301–312. doi: 10.1016/j.ydbio.2008.12.011

Bian, Q., Qian, J., Xu, L., Chen, J., Song, L. and Wang, X. (2006). The toxic effects of 4-tert-octylphenol on the reproductive system of male rats. *Food and Chemical Toxicology*, **44**(8), 1355–1361. doi: 10.1016/j.fct.2006.02.014

Grasso, M., Fuso, A., Dovere, L., De Rooij, D. G., Stefanini, M., Boitani, C. and Vicini, E. (2012). Distribution of GFRA1-expressing spermatogonia in adult mouse testis. *Reproduction*, **143**(3), 325–332. doi: 10.1530/REP-11-0385

Guan, Y., Liang, G., Martin, G. B. and Guan, L. L. (2017). Functional changes in mRNA expression and alternative pre-mRNA splicing associated with the effects of nutrition on apoptosis and spermatogenesis in the adult testis. *BMC Genomics*, **18**(1), 64. doi: 10.1186/s12864-016-3385-8

Gustafson, E. A. and Wessel, G. M. (2010). Vasa genes: Emerging roles in the germ line and in multipotent cells. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, **32**(7), 626–637. doi: 10.1002/bies.201000001

Hashemi Karoii, D. and Azizi, H. (2022). A review of protein–protein interaction and signaling pathway of vimentin in cell regulation, morphology and cell differentiation in normal cells. *Journal of Receptors and Signal Transduction*, 1–9.

Hashemi Karoii, D., Azizi, H. and Skutella, T. (2022). Microarray and in silico analysis of DNA repair genes between human testis of patients with nonobstructive azoospermia and normal cells. *Cell Biochemistry and Function*, **40**(8), 865–879. doi: 10.1002/cbf.3747

Kanatsu-Shinohara, M., Ogonuki, N., Matoba, S., Morimoto, H., Shiromoto, Y., Ogura, A. and Shinohara, T. (2022). Regeneration of spermatogenesis by mouse germ cell transplantation into allogeneic and xenogeneic testis primordia or organoids. *Stem Cell Reports*, **17**(4), 924–935. doi: 10.1016/j.stemcr.2022.02.013

Kanbar, M., Vermeulen, M. and Wyns, C. (2021). Organoids as tools to investigate the molecular mechanisms of male infertility and its treatments. *Reproduction*, **161**(5), R103–R112. doi: 10.1530/REP-20-0499

Kang, K., Niu, B., Wu, C., Hua, J. and Wu, J. (2020). The construction and application of lentiviral overexpression vector of goat miR-204 in testis. *Research in Veterinary Science*, **130**, 52–58. doi: 10.1016/j.rvsc.2020.02.014

Karoii, D. H., Azizi, H. and Amirian, M. (2022). Signaling pathways and protein–protein interaction of vimentin in invasive and migration cells: A review. *Cellular Reprogramming*, **24**(4), 165–174. doi: 10.1089/cell.2022.0025

Khadivi, F., Koruji, M., Akbari, M., Jabari, A., Talebi, A., Ashouri Movassagh, S., Panahi Boroujeni, A., Feizollahi, N., Nikmahzar, A., Pourahmadi, M. and Abbasi, M. (2020). Application of platelet-rich plasma (PRP) improves self-renewal of human spermatogonial stem cells in two-dimensional and three-dimensional culture systems. *Acta Histochemica*, **122**(8), 151627. doi: 10.1016/j.acthis.2020.151627

Li, H., Liang, Z., Yang, J., Wang, D., Wang, H., Zhu, M., Geng, B. and Xu, E. Y. (2019). DAZL is a master translational regulator of murine spermatogenesis. *National Science Review*, **6**(3), 455–468. doi: 10.1093/nsr/nwy163

Marcon, L., Zhang, X., Hales, B. F., Robaire, B. and Nagano, M. C. (2011). Effects of chemotherapeutic agents for testicular cancer on rat spermatogonial stem/progenitor cells. *Journal of Andrology*, **32**(4), 432–443. doi: 10.2164/jandrol.110.011601

https://doi.org/10.1017/S0967199423000047 Published online by Cambridge University Press

Moraveji, S. F., Esfandiari, F., Sharbatoghli, M., Taleahmad, S., Nikeghbalian, S., Shahverdi, A. and Baharvand, H. (2019). Optimizing methods for human testicular tissue cryopreservation and spermatogonial stem cell isolation. *Journal of Cellular Biochemistry*, **120**(1), 613–621. doi: 10.1002/jcb.27419

Niazi Tabar, A., Azizi, H., Hashemi Karoii, D. and Skutella, T. (2022a). Testicular localization and potential function of vimentin positive cells during spermatogonial differentiation stages. *Animals: An Open Access Journal from MDPI*, **12**(3), 268. doi: 10.3390/ani12030268

Niazi Tabar, A. N., Sojoudi, K., Henduei, H. and Azizi, H. (2022b). Review of Sertoli cell dysfunction caused by COVID-19 that could affect male fertility. *Zygote*, **30**(1), 17–24. doi: 10.1017/S0967199421000320

Onohara, Y., Fujiwara, T., Yasukochi, T., Himeno, M. and Yokota, S. (2010). Localization of mouse vasa homolog protein in chromatoid body and related nuage structures of mammalian spermatogenic cells during spermatogenesis. *Histochemistry and Cell Biology*, **133**(6), 627–639. doi: 10.1007/s00418-010-0699-5

Rahmani, F., Movahedin, M., Mazaheri, Z. and Soleimani, M. (2019). Transplantation of mouse iPSCs into testis of azoospermic mouse model: *In vivo* and *in vitro* study. *Artificial Cells, Nanomedicine, and Biotechnology*, **47**(1), 1585–1594. doi: 10.1080/21691401.2019.1594854

Rezaei Topraggaleh, T., Rezazadeh Valojerdi, M., Montazeri, L. and Baharvand, H. (2019). A testis-derived macroporous 3D scaffold as a platform for the generation of mouse testicular organoids. *Biomaterials Science*, **7**(4), 1422–1436. doi: 10.1039/c8bm01001c

Richer, G., Baert, Y. and Goossens, E. (2020). In-vitro spermatogenesis through testis modelling: Toward the generation of testicular organoids. *Andrology*, **8**(4), 879–891. doi: 10.1111/andr.12741

Sakib, S., Voigt, A., Goldsmith, T. and Dobrinski, I. (2019). Three-dimensional testicular organoids as novel *in vitro* models of testicular biology and toxicology. *Environmental Epigenetics*, **5**(3), dvz011. doi: 10.1093/eep/dvz011

Sakib, S., Goldsmith, T., Voigt, A. and Dobrinski, I. (2020). Testicular organoids to study cell–cell interactions in the mammalian testis. *Andrology*, **8**(4), 835–841. doi: 10.1111/andr.12680

Shi, Y. and Ai, W. (2013). Function of KLF4 in stem cell biology. In: Bhartiya, D. and Lenka, N. (eds). *Pluripotent Stem Cells*. doi: 10.5772/54370

Shukalyuk, A. I., Golovnina, K. A., Baiborodin, S. I., Gunbin, K. V., Blinov, A. G. and Isaeva, V. V. (2007). Vasa-related genes and their expression in stem cells of colonial parasitic rhizocephalan barnacle *Polyascus polygenea* (Arthropoda: Crustacea: Cirripedia: Rhizocephala). *Cell Biology International*, **31**(2), 97–108. doi: 10.1016/j.cellbi.2006.09.012

Strange, D. P., Zarandi, N. P., Trivedi, G., Atala, A., Bishop, C. E., Sadri-Ardekani, H. and Verma, S. (2018). Human testicular organoid system as a novel tool to study Zika virus pathogenesis. *Emerging Microbes and Infections*, **7**(1), 82. doi: 10.1038/s41426-018-0080-7

Szklarczyk, D., Gable, A. L., Nastou, K. C., Lyon, D., Kirsch, R., Pyysalo, S., Doncheva, N. T., Legeay, M., Fang, T., Bork, P., Jensen, L. J. and von Mering, C. (2021). The STRING database in 2021: Customizable protein–protein networks, and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Research*, **49**(D1), D605–D612. doi: 10.1093/nar/gkaa1074

Turnbull, C., Rapley, E. A., Seal, S., Pernet, D., Renwick, A., Hughes, D., Ricketts, M., Linger, R., Nsengimana, J., Deloukas, P., Huddart, R. A., Bishop, D. T., Easton, D. F., Stratton, M. R., Rahman, N. and UK Testicular Cancer Collaboration. (2010). Variants near DMRT1, tert and ATF7IP are associated with testicular germ cell cancer. *Nature Genetics*, **42**(7), 604–607. doi: 10.1038/ng.607

Wei, Y., Yang, D., Du, X., Yu, X., Zhang, M., Tang, F., Ma, F., Li, N., Bai, C., Li, G. and Hua, J. (2021). Interaction between DMRT1 and PLZF protein regulates self-renewal and proliferation in male germline stem cells. *Molecular and Cellular Biochemistry*, **476**(2), 1123–1134. doi: 10.1007/s11010-020-03977-3

Yamaguchi, S., Kimura, H., Tada, M., Nakatsuji, N. and Tada, T. (2005). Nanog expression in mouse germ cell development. *Gene Expression Patterns*, **5**(5), 639–646. doi: 10.1016/j.modgep.2005.03.001

Zheng, Y., Feng, T., Zhang, P., Lei, P., Li, F. and Zeng, W. (2020). Establishment of cell lines with porcine spermatogonial stem cell properties. *Journal of Animal Science and Biotechnology*, **11**, 33. doi: 10.1186/s40104-020-00439-0

https://doi.org/10.1017/S0967199423000047 Published online by Cambridge University Press