Evaluation Of Porcine Somatic And Testicular Germ Cells During Differentiation And Proliferation

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Abstract

Background: Spermatogenesis is the complex process of sperm production to transmit paternal genetic information to the subsequent generation. This process is determined by the collaboration of several germ and somatic cells, most importantly spermatogonia stem cells and Sertoli cells. To characterize germ and somatic cells in the tubule seminiferous contort in porcine and consequently has an impact on the analysis of porcine fertility.

Materials and methods: Germ cells were extracted from porcine testis by enzymatic digestion before being expanded on Sandos inbred mice (SIM) embryo-derived thioguanine and ouabain-resistant fibroblasts (STO) feeder layer supplemented with FGF, EGF, and GDNF. Immunohistochemistry (IHC) and immunocytochemistry (ICC) analysis for Sox9, Vimentin, and PLZF markers were performed to examine the generated colonies of porcine testicular cells. Electron microscopy was also utilized to analyze the morphological features of the extracted porcine germ cells.

Results: IHC analysis revealed that Sox9 and Vimentin were expressed in the basal compartment of the seminiferous tubules. Moreover, ICC results showed that the cells have low expression of PLZF while expressing vimentin. Heterogeneity of the in vitro cultured cells was detected via morphological analysis by the electron microscope.

Conclusion: In this experimental study, we tried to reveal the exclusive information which obviously could be helpful for future success in achievement to proper therapies against infertility and sterility as an important global issue.

Plain English Summary

Spermatogenesis is a critical stage in male fertility that takes place in seminiferous epithelial. Together with Sertoli cells, male germ cells are the key players in spermatogenesis, the complicated biological process of developing male gametes from diploid spermatogonia that results in sperm production. During the final stage of spermatogenesis, mature spermatids are released from the supporting Sertoli cells into the lumen of the seminiferous tubules. In this experimental research, we performed morphology characterization by Electron microscopy, analysis of protein and gene expression by immunocytochemistry, immunohistochemistry, Fluidigm Real-Time polymerase chain reaction (RT-PCR) and flow cytometry to characterization of different cells including germ cells and somatic cells in the pig seminiferous tubule. Immunohistochemistry analysis revealed that Sox9 and Vimentin were expressed in the basal compartment of the seminiferous tubules. Moreover, immunocytochemistry results showed that the cells have low expression of PLZF while expressing vimentin. Heterogeneity of the in vitro cultured cells was detected via morphological analysis by the electron microscope. thus, improvements in spermatogonial stem cells isolation and culture would be helpful in future clinical treatments to solve the reproductive problems of families influenced by infertility.

Introduction

Spermatogenesis is a fundamental part of natural reproduction [1] that occurs as a complex and regulated growth process [2] in the epithelium of testicular seminiferous tubules [3]. This process greatly depends on the proper functioning of the spermatogonia stem cells (SSCs), which initiate and maintain the continuous process of sperm production and differentiation [1, 3, 4]. All stages of spermatogenesis are supported by Sertoli cells [5], in other words, sperm development is followed by nutrition by Sertoli cells, which are the somatic cells of the seminiferous tubules [6] that have evolved from the spermatogonia to the central lumen during evolution. Thus, Sertoli cells play an important role in spermatogenesis by regulating the growth of spermatogonia cells and supporting spermatogonia stem cells [7].

Sertoli cells regulate and maintain Spermatogonia stem cells (SSCs) by regulating markers including Sox9 (SRY-Box Transcription Factor 9) and VIMENTIN. SOX9 is a major transcription factor in the sox protein family and regulates many stem cell development programs [8, 9]. Sox (SRY-related HMG-box) proteins are generally involved in the growth process, and among the sox family, sox9 differentiates the testicles [10]. Vimentin is a major mesenchymal protein and one of the primary Sertoli cells' markers that also appears to be an important regulatory factor [11, 12].

The SSCs responsible for maintaining spermatogenesis [4] are located on the basement membrane of the seminiferous tube [13] and account for less than 0.03% of the testicular cells [14] but have the potential to self-renew [13] and differentiate indefinitely [15]. Thus, SSCs are crucial in transmitting genetic information to the next generation through the continuous production of spermatogonia and their differentiation into haploid spermatids [4, 16].

Some markers, such as promyelocytic leukaemia zinc finger (PLZF), are produced by germ cells and are active in developing these cells. PLZF is essential for the self-renewal, reproduction, and maintenance of SSCs [17], and consequently, for the maintenance of spermatogonia. Removal of PLZF has been shown to lead to spermatogonia discharge, resulting in a phenotype similar to "testicular ageing" [18].

SSCs can be cultured in special in-vitro conditions with the help of feeder layers [19], which are the main element in the culture and growth of spermatogonia stem cells [20]. Use of Sandos inbred mice (SIM) embryo-derived thioguanine and ouabain-resistant fibroblasts or STO feeder cells is a common method for the in-vitro culture of the pluripotent stem cells [21]. Similarly, the STO feeder layer is the most suitable option for a stable in-vitro culture of spermatogonia stem cells for long periods [19].

This experimental study examined the localization of the essential markers including Sox9, Vimentin, and PLZF by immunohistochemical (IHC) and immunocytochemical (ICC) analysis of porcine testicular tissue sections and cells after isolation and expansion on the STO feeder layer.

In this study, we use porcine testis as biologic model to introduce reliable germ cell marker which could be helpful for future studies for diagnosis of male infertility and also reproductive biology advances.

Methods

Study Population

Four pairs of testes from euthanized porcine with an average $\pm$ SD of 8.0 ± 2.0 weeks were obtained from the Institute of Anatomy and Cell Biology, Medical Faculty, University of Heidelberg, Germany. This study was approved by the ethical committee of Amol University of special modern technologies (Ir.ausmt.rec.1398.03.07) and carried out according to the MOU agreement between Amol University of Special Modern Technologies and the University of Heidelberg between April 2020 and March 2021.

Digestion And Primary Culture Of Testicular Cells

Testes were removed surgically and placed in phosphate buffer solution (PAA LABORATORIES GMBH, USA). After decapsulation, undigested tissue were finely minced and digested in a solution containing collagenase A (0.5 mg/ml) (Sigma Aldrich), Dispase (0.5 mg/ml) (Sigma Aldrich) and DNase (0.5mg/ml) (Sigma Aldrich) in 37° C water bath for 15 min. The remaining tissue was further digested in a solution containing collagenase A (1 mg/ml) (Sigma Aldrich), trypsin (0.5 mg/ml) (Sigma Aldrich), DNase (0.5mg/ml) (Sigma Aldrich) and hyaluronidase type v (Invitrogen) in the same water bath conditions for 20 min. Isolated cells were centrifuged at 400g for 10 min and re-suspended in DMEM (PAA LABORATORIES GMBH, USA) + 10% FBS (Invitrogen) and placed in GSCs culture dishes containing 1% L-glutamine (PAA LABORATORIES GMBH, USA), 6 mg/ml D + glucose (Sigma Aldrich, USA), StemPro-34 medium, 1% penicillin/streptomycin (PAA LABORATORIES GMBH, USA), 5µg/ml bovine serum albumine (Sigma Aldrich, USA), 1% N2-supplement (Invitrogen, USA), 60 ng/ml progesterone (Sigma Aldrich, USA), 0,1% β-mercaptoethanol (Invitrogen, USA), 8 ng/ml GDNF (Sigma Aldrich, USA), 1% MEM vitamins (PAA LABORATORIES GMBH, USA), 30 ng/ml estradiol (Sigma Aldrich, USA), 1% non-essential amino acids (PAA LABORATORIES GMBH, USA), 20 ng/ml epidermal growth factor (EGF), 10 ng/ml FGF (Sigma Aldrich, USA), 100 U/ml human LIF (Millipore), 100 µg/ml ascorbic acid (Sigma Aldrich, USA), (Sigma Aldrich, USA), 30 µg/ml pyruvic acid (Sigma Aldrich, USA), 1% ES cell qualified FBS, and 1 µl/ml DL-lactic acid (Sigma Aldrich, USA) at 37°C and 5% CO₂ in air [23, 24].

Tissue Processing For Immunohistochemical Staining

Testicular tissue was decapsulated of tunica albuginea, washed with PBS, and fixed in 4% paraformaldehyde. Next, 8–10 µm thick sections were prepared using a microtome (BRAND, COUNTRY). The sections were mounted on Hydrophilic Plus slides and stored at room temperature until used. For the immunohistochemical staining process, slides were washed by xylene before being placed in a series of decreasing concentrations of ethanol in water. Before staining, antigen retrieval was done by heat-induced epitope retrieval (HIER) methods at 95°C for 20 minutes, and the non-specific binding sites in the tissue sections were blocked with 10% serum/0.3% Triton in PBS. The prepared slides were divided into two groups and incubated with either primary sox9 antibody or vimentin antibody (Sigma) and species-

specific secondary antibodies. The characterization of labelled cells was analyzed by confocal laser scanning microscope Zeiss LSM (Zeiss, Germany)[20].

Immunocytochemical Staining

In this process, PLZF and vimentin (Sigma) were used as primary antibodies. Isolated cells from the testis were fixed, permeabilized and blocked with 4% paraformaldehyde and 0.1% Triton X-100 /PBS and 1% BSA/PBS before being incubated with the primary antibodies. The process was followed by overnight incubation (usually ~ 16hs) with fluorochrome species-specific secondary antibody at 4°C. The labelled cells were identified by simple nuclear counterstain with 0.2 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) dye. Labelled positive cells with antibodies were considered by a confocal laser scanning microscope Zeiss LSM 700, and images of cells were obtained using a Zeiss LSM-TPMT camera [20, 23, 24].

Electron Microscopy

Cultured cells were centrifuged in 15 ml centrifuge tubes. The pellets were fixed with paraformaldehyde/glutaraldehyde in PIPES; the fixative was renewed after five minutes. Pellets were then post-fixed in OsO₄/potassium hexacyanoferrate for 50 min, rinsed in a solution of uranyl acetate buffer and sodium maleate buffer (pH 6.0), and block-stained with uranyl acetate. Following the dehydration through increasing concentrations of ethanol (5 min each, 4 x 20 min), the tissue was Epon-embedded (polymerization at 60°C, 24 h). Ultrathin sections (50 nm) were examined with a Zeiss EM10.

Results

The localization of SOX9 and VIMENTIN in porcine testicular sections assessed by immunohistochemical examinations can be seen in (Fig. 1A, B). As expected, the expression of SOX9 and VIMENTIN was observed near the basal membrane where the Sertoli cells are located. SOX9 and VIMENTIN were expressed only in some areas of the Sertoli cells (Fig. 1A2, B2). We used DAPI staining to observe the expression of these markers, which is used to identify the cell's nucleus.

Figure 2 demonstrates the growth of cell colonies that were observed spread on the STO feeder layer. Examination of bright-field microscopy images suggests that the colonies that appear in clusters to be spermatogonia stem cells.

Results of immunocytochemical examinations of the expression of VIMENTIN and PLZF in porcine testicular sections can be seen in (Fig. 3A, B). The PLZF protein was expressed in the basement membrane of seminiferous tubules (Fig. 3B1), while VIMENTIN expression was observed in the cytoplasm sections (Fig. 3A1). This observation revealed that in addition to the low expression of *PLZF*, the seminiferous tubule cells could also express vimentin.

Examining the electron microscopic images revealed heterogeneous and different sizes of the nuclei (Fig. 4). In some cells, the nucleus was so large that it took up the entire cytoplasmic space (Fig. 4B1), while it was much smaller in the others (Fig. 4A2). The nucleus was located close to the cell membrane in some cells (Fig. 4A1, A2, B3) and the centre in some others (Fig. 4B2). Also, the black parts seen in the electron microscopy image included the nucleus, organs, and vesicles, and in the nucleus, dense nucleus and heterochromatin and euchromatin were seen. In the cytoplasm, multivesicular bodies were observed near the Golgi apparatus, and the Golgi apparatus are scattered at the basal and lateral levels and generally throughout the cytoplasm.

Discussion

This study utilized immunohistochemistry analysis for Sox9 and Vimentin expression in the seminiferous tubules of porcine and observed expression of sox9 and vimentin in the base membrane of the seminiferous tubule. Kirschner and colleagues previously investigated the effect of hypoxia on the transcription of the SOX9 gene under the control of a protein called Wilms tumor protein1 (WT1) by immunohistochemistry analysis of SOX9 gene expression in the testicles in the male murine embryonic gonads, demonstrating that hypoxia reduced the transcription of SOX9 in the cultured testicles [22].

This study also assessed bright-field images of germ cells' expansion on the STO feeder layer, supplemented with Fibroblast growth factor (FGF), Epidermal growth factor (EGF), and Glial cell line-derived neurotrophic factor (GDNF). A similar study by Tlapakova et al. [23] on amphibian testicular cell cultures observed small colonies of ES-like cells on the Sertoli-like feeder layer two months after the experiment, suggesting that, unlike higher vertebrates, SOX9 is expressed in the oocyte cytoplasm indicating its role in testicular differentiation and not in gender determining. On the other hand, Immunohistochemical analysis showing a strong expression of vimentin in transplanted cells, as well as a majority of cells expressing Vimentin and SOX9 simultaneously, indicated the existence of a common progenitor of Sertoli cell [23].

Monfared and colleagues investigated the effect of the environment of adult Sertoli cells on the induction of germ cells from the mesenchymal stem cells of the mouse bone marrow. In this study, Sertoli cells have been isolated from germ cells, and after evaluation, the presence of vimentin protein in the cytoplasm of Sertoli cells has been detected by immunocytochemistry analysis [24].

In this study, the immunocytochemical analysis demonstrated the expressions of both vimentin and *PLZF*. A reason for vimentin's expression could be that undifferentiated spermatogonia stem cells have a large nucleus and a small cytoplasm. However, as the spermatogonia stem cells differentiate, their nucleus gets gradually smaller while their cytoplasm grows larger. Vimentin is an intermediate protein essential for maintaining the stability of spermatogonia stem cells, which can explain the expression of vimentin during differentiation, further leading to increased *PLZF* expression.

A similar study by Tang et al. in association with *PLZF* expression pattern in testicular sections in mice by Immunohistochemistry (IHC) and Immunofluorescence (IF) has reported *PLZF* as a classic

spermatogonia biomarker widely expressed in subspecies of spermatogonia, with its performance having been confirmed in animal genetic models and transplant methods, [25].

Besides, several recent studies have identified PLZF as a tumour suppressor in cancer progression, also acting as an oncoprotein in certain types of cancer (REF). While some preliminary studies in stem cells have linked PLZF to haematological malignancies, it has been shown that PLZF is also expressed in many cell types, including the prostate, and to a large extent in its lumen cells and involved in various tumours. Immunohistochemical analysis also showed strong PLZF expression in benign and low-grade prostate tumours, but no significant change in PLZF expression in benign prostate versus malignant prostate has been observed [26].

For example, immunohistochemistry analysis of the expression of PLZF in prostate cancer has shown exclusive expression of PLZF in the lumen cell nucleus and its absence in basal cells, highlighting its importance as an indicator [27].

In another study on a tumour suppressor caused by androgen and, in turn, inhibited by ADT, the tissue of a patient with high-grade prostate cancer was negatively associated with PLZF expression. In that study, the mRNA PLZF expression was very low in tumours where the PLZF was removed, emphasizing the importance of the signalling pathways in prostate cancer as regulators of PLZF expression. The mentioned study showed that a decrease in PLZF accelerated the proliferation, survival, migration, and invasion of human prostate cancer cells, and an increase in PLZF could be a strategy for suppressing prostate cancer metastasis as well as tumour growth [28].

During a recent experiment conducted by Chen et al., it was stated that gallbladder cancer is a fatal and common cancer of the bile duct and that PLZF inhibits the growth of GBC cells in vitro. Chen et al. used IF and IHC to detect the function, expression, and overexpression of PLZF in a xenograft mice model. The study provided evidence that in vitro and in vivo PLZF acts as a potent inhibitor of tumour growth and GBC liver metastasis and that PLZF expression in GBC is significantly reduced [29].

In this study, we analyzed the morphology of the germ cells after expansion and isolation by electron microscopy. A previous study on porcine mesenchymal stem cells identified the subunits of mesenchymal stem cells (MSC) using nanoparticle tracking analysis, flow cytometry, and transmission electron microscopy. This study illustrated the differences between populations of extracellular vesicles derived from MSCs and evaluated quantitative analysis for published phenotypic descriptions of mesenchymal stem cells and gene expression, and that's for study the morphology of EVs derived from MSC, they were transferred to an electron microscope after staining and then compared [30].

Electron microscopy assessment of mouse germ cells survival in isolated gonads suggested cellular adhesion between Sertoli cells and germ cells as an important factor for the survival of germ cells [31].

An experiment on mice with a conditional knockout allele for the PLZF factor, created using Crispr/Cas9 engineering and gene editing, showed that PLZF mediated a wide range of biological processes and

confusion in its function led to countless physiological defects and removing PLZF takes into account its performance. Thus, due to the remarkable speed and accuracy, Crispr genome engineering has been used to produce the conditional elimination allele PLZF in mice, and it was stated that the PLZF/f mice are a valuable tool for better describing the function of PLZF in specific tissues and cells [32].

IHC analysis of microRNA expression during the differentiation of cells derived from the mesenchymal stem cells of the mouse bone marrow showed that microRNAs play an important role in gene expression at the level after transcription [33]. this study also evaluated the expression of PLZF protein in PGC-like cells and SSC-like cells, and has been pointed out that the expression of markers was examined using related antibodies and during immunocytochemistry staining, bone marrow mesenchymal stem cells were transferred to the fluorescent microscope for observation after washing and addition of antibodies and incubation, and finally dying of the cell nucleus [33].

Conclusion

In the present study, we showed expression pattern of important genes and proteins in reproduction system to reveal the exclusive information which obviously could be helpful for future success in achievement to proper therapies against infertility and sterility as an important global issue.

Declarations

Acknowledgements

No applicable.

Authors' Contributions

Amirreza Niazi Tabar: Carried out and design the experiment, assembly of data and data analysis, wrote the manuscript and data analysis; Hossein Azizi.; Carried out and provided critical feedback; Mehdi Mehdinezhad Roshan: Data analysis and Edited the manuscript; Hiva Alipour: data analysis and Edited the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The data sets analyzed for the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Amol University of Special Modern Technologies (Ir.ausmt.rec.1398.03.07).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Immunohistochemical characterization in testis sections in porcine, A. Immunohistochemistry for the expression of SOX9, A1. Merged image for expression of SOX9 and DAPI staining, A2. Green parts for the SOX9 expression, A3. Blue parts for DAPI staining. B. Immunohistochemistry for the expression of SOX9 and VIMENTIN, B1. Merged image for green parts for expression of SOX9 and red parts for expression of

VIMENTIN and blue parts for DAPI staining, B2. Green parts for the SOX9 expression, B3. Red parts for the VIMENTIN expression (Scale bar = 50 μ m).

Figure 2

The expansion of porcine testicular germ cells on the STO feeder layer.

Figure 3

Immunocytochemical characterization in the cell culture of porcine, A. Immunocytochemistry for the expression of VIMENTIN, A1. Merged image for expression of VIMENTIN and DAPI staining, A2. Red parts for the VIMENTIN expression, A3. Blue parts for DAPI staining. B. Immunocytochemistry for the expression of PLZF, B1. Merged image for expression of PLZF and DAPI staining, the arrows show the expression of the related protein in the seminiferous tubule. B2. Green parts for the PLZF expression, B3. Blue parts for DAPI staining (Scale bar = 50 μm).

Figure 4

The electron microscopy of cultured cells shows the characteristics of the cells and the different sizes of the nucleus.