

## Characterization and Isolation of Ovine Spermatogonial Stem Cells

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**Abstract:** Identification and isolation of Spermatogonial Stem Cells (SSCs) are a prerequisite for culture, genetic manipulation and transplantation research. In this study, the appropriate age of the donor ram for SSCs isolation was investigated. Spermatogonial stem cell specific marker protein Promyelocytic Leukaemia Zinc-Finger (PLZF) and male germ cell marker VASA double staining were checked on the paraffin sections of ovine testis. Type A spermatogonial marker PGP9.5 and sertoli cell marker Vimentin double staining were also used to confirm testes development. Results indicated that testes from neonatal rams had only one type of undifferentiated germ cell gonocyte and should be suitable for ovine SSCs isolation. VASA and Vimentin double staining of the isolated testis cells confirm the efficiency of ovine SSCs isolation.

**Key words:** Ovine, testis, SSCs, fluorescent staining, Vimentin double staining, China

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### INTRODUCTION

In moving toward identifying spermatogonial stem cells in rams, testicular cell types (spermatogonia, spermatogonial stem cells, sertoli cells, myoid cells and leydig cells) need to be first characterized. Many investigations have identified testis cell populations using surface markers. Application of c-kit marker for isolation of spermatogonia results in the selection of more differentiated spermatogonia than spermatogonial stem cells (Shinohara and Brinsler, 2000). Isolation of mouse spermatogonia on the basis of  $\alpha$ -6 and  $\beta$ -1 integrin markers resulted in an enriched population of SSCs (Shinohara *et al.*, 1999). Expression of GFR $\alpha$ -1, Ret, Egr3 and EpCAM have been used as SSCs markers (Meng *et al.*, 2000; Tadokoro *et al.*, 2002; Naughton *et al.*, 2006; Hamra *et al.*, 2004). As type A spermatogonia marker for bovine, DBA has been reported not to bind to any type of cell in prepubertal ovine testis (Ertl and Wrobel, 1992; Izadyar *et al.*, 2002; Rodriguez-Sosa *et al.*, 2006). PGP9.5 has been used to identify type A spermatogonia in monkey, bovine and ovine testis (Tokunaga *et al.*, 1997, 1999; Wrobel *et al.*, 1996; Wrobel, 2000; Rodriguez-Sosa *et al.*, 2006; Herrid *et al.*, 2009). Pomyelocytic Leukaemia Zinc-Finger (PLZF) was used as a specific marker for gonocytes and spermatogonial stem cells in ram testis (Borjigin *et al.*, 2010).

The aims of the present study were to confirm the appropriate age of the donor ram for SSCs isolation. PLZF

and VASA double fluorescent staining were checked on the paraffin sections of four stages of ovine testis. In addition, PGP9.5 and Vimentin were used to quantify changes in tubular cells during development. Subsequently, VASA and Vimentin double staining were used to evaluate the efficiency of ovine SSCs isolation.

### MATERIALS AND METHODS

**Animals:** Merino rams were selected to provide testis samples representing four stages of testis development: Stage 1, the neonatal period (1-2 months old) Stage 2, the peripubertal period (3-4 months old) Stage 3, the prepubertal period (5-6 months old) and Stage 4, the post-pubertal period (9-10 months old). Three animals were used for each age group. Animals were handled and treated according to the guidelines of the Animal Ethics Committee at Inner Mongolia University, Hohhot.

**Collection of testis samples:** Rams were castrated under general anaesthesia (0.1 mg kg<sup>-1</sup> xylazine followed by 3 mg kg<sup>-1</sup> ketamine). Antibiotics and an analgesic/anti-inflammatory were administered postoperatively. After castration, the testes were washed in Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma-Aldrich). The tunica albuginea, epididymides and excess connective tissue were removed and testis weight was recorded. A sample for immunohistochemistry was collected from the equatorial region of one testis from each animal, fixed for

6 h in modified Davidson's fixative (MD; 10% formalin, 15% ethanol, 5% acetic acid) and then transferred to 70% ethanol (Sigma-Aldrich). Each tissue was dehydrated through a graded ethanol series (70, 80, 90 and 100%; 2×1 h each) and xylene (2×1 h; Sigma-Aldrich) infiltrated with paraffin (60°C; 4×1 h) before being embedded in paraffin wax. Sections (5 µm) were cut and dried overnight on glass slides at 37°C and stored at room temperature.

**Double fluorescent staining of testis tissues with PLZF-VASA or PGP9.5-Vimentin:** After antigen retrieval, all slides were treated with 10% newborn bovine serum (NBS; Invitrogen, Carlsbad, CA, USA) in TBS for 30 min to block non-specific adhesion sites. Sections were then incubated with a mixture of mouse anti-PLZF (1:100) and rabbit anti-VASA (1:10) or mixture of rabbit anti-PGP9.5 (1:200) and mouse anti-Vimentin (1:200) for 30 min. After incubation, slides were rinsed in TBST and a mixture of chicken anti-rabbit Alexa fluor 488 (1:200; Invitrogen) and goat anti-mouse Alexa fluor 594 (1:200; Invitrogen) in 0.5% Bovine Serum Albumin (BSA) in TBS was applied for 30 min before slides were again rinsed in TBS and mounted in Prolong Gold (Invitrogen).

**Enzymatic isolation of testis germ cells:** A two-step enzymatic isolation and differential plating procedure was used to obtain individual testicular cells and enrich gonocytes as described by Borjigin *et al.* (2010). Briefly, testis tissues were placed in a tea strainer and ground with a 5 mL syringe plunger. The remaining tubule section was incubated with collagenase (1 mg mL<sup>-1</sup>; Type IV; Sigma-Aldrich) in a shaking water bath at 37°C. During this time, the tissue samples were frequently monitored under microscopy and the reaction was stopped when individual tubules were observed then treated with trypsin (2.5 mg mL<sup>-1</sup>; Gibco-BRL) in PBS for 5-10 min at 37°C. DNase I (7 mg mL<sup>-1</sup>; Sigma, St. Louis, MO, USA) in DMEM was added 1 min after trypsin treatment. An equal volume of heat-inactivated fetal bovine serum (FCS; Invitrogen) was used to inactivate the trypsin digestion. The resultant cell suspension was then filtered through a cell strainer and centrifuged at 1500 g for 5 min at room temperature. The pellets were resuspended in 10 mL DMEM containing 5% FBS at a density of 40-80×10<sup>6</sup> cells mL<sup>-1</sup>. Cell viability was assessed by Trypan blue exclusion.

**Double fluorescent staining of the isolated cells smears:** Smears were prepared from freshly isolated cells and dried on a warm stage at 37°C. The slides then were cooled at room temperature for 30 min and wrapped in foil and stored at -80°C until staining. Prior to being stained, slides were brought to room temperature before being

unwrapped to prevent condensation. Smears were fixed in Bouin's solution for 2 min, rinsed thoroughly with TBST then incubated with a mixture of incubated with a mixture of rabbit anti-VASA (1:10) and mouse anti-Vimentin (1:200) for 30 min. After incubation, slides were rinsed in TBST and a mixture of chicken anti-rabbit Alexa fluor 488 (1:200; Invitrogen) and goat anti-mouse Alexa fluor 594 (1:200; Invitrogen) in 0.5% Bovine Serum Albumin (BSA) in TBS was applied for 30 min before slides were again rinsed in TBS and mounted in Prolong Gold (Invitrogen).

## RESULTS AND DISCUSSION

**Immunolocalisation of PLZF and VASA in ovine testis:** Micrographs of immunostained testis sections of each stage are shown in Fig. 1. The coincidences seen in merged images from double staining of testis sections with PLZF and VASA demonstrated that PLZF immunoreactivity was localised in the nuclei of gonocytes or spermatogonia whereas immunostaining for VASA was seen in the cytoplasm of these cells. At Stage 1, most of the VASA-positive cells also stained positive to PLZF antibody (Fig. 1a). However, at Stage 2 about 1/2 of the VASA-positive cells were negative for PLZF expression (Fig. 1b). After that VASA-positive cell increased dramatically at stage 3-4 whereas PLZF and VASA double positive cell become very rare (Fig. 1c and d).

**Immunolocalisation of PGP9.5 and Vimentin in ovine testis:** Antibodies against DBA and PGP9.5 were used to

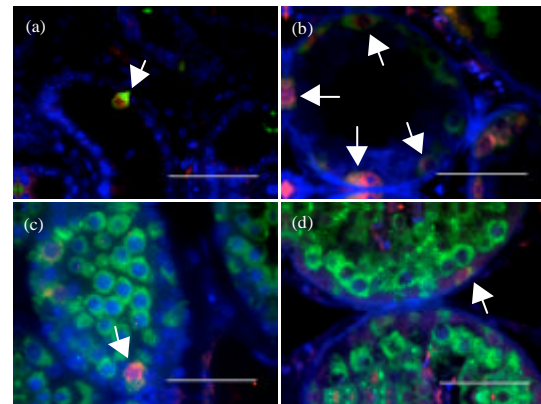


Fig. 1: Fluorescence double staining of sheep testis sections for PLZF and VASA at; a) Stage 1; b) Stage 2; c) Stage 3 and d) Stage 4. Red indicates PLZF-positive cells; green indicates VASA-positive cells. Blue indicates 4'-6'-diamidino-2-phenylindole staining of the nuclei. Arrows indicate coexpression of PLZF and VASA. Scale bar = 50 µm

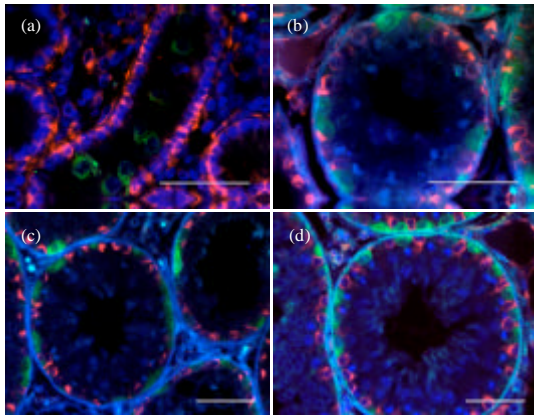


Fig. 2: Fluorescence double staining of sheep testis sections for PGP9.5 and Vimentin at; a) Stage 1; b) Stage 2; c) Stage 3 and d) Stage 4. Red indicates Vimentin-positive cells; green indicates PGP9.5-positive cells. Blue indicates 4'-6'-diamidino-2-phenylindole staining of the nuclei. Scale bar = 50  $\mu$ m

label spermatogonia whereas antibodies against vimentin and GATA-4 were used to identify sertoli cells (Wrobel, 2000). Double staining of testis sections with PGP9.5 and Vimentin demonstrated that PGP9.5-positive cell is not Vimentin-positive. Vimentin-positive cell had irregular cell type and PGP9.5-positive cell was round or oval shape (Fig. 2a-d).

#### Isolation efficiency of the neonatal ram testis germ cell:

Isolation of tissues from Stage 1 is expected to yield relatively higher numbers of gonocytes or SSCs due to the lack of an advanced germ cell population in the seminiferous tubules. The results of the comparison of isolation efficiency showed that most VASA positive cells in the tubular sections ( $7.3 \pm 0.1$ ) were recovered in the cell suspensions ( $7.2 \pm 0.8$ ) from Stage 1 testes (Fig. 3).

Previous report (Borjigin *et al.*, 2010) has shown that PLZF-positive cells in ovine testis are a subpopulation of type A spermatogonia and thus likely represent the undifferentiated spermatogonia including SSCs with the ability to self-renew. The distribution pattern of the PLZF-positive cells observed in this study is consistent with gonocyte migration and spermatogonia location in the seminiferous tubules of neonatal, peripubertal, prepubertal and post-pubertal ovine testes and these results are similar to the expression pattern of PLZF in mouse testis (Buaas *et al.*, 2004; Costoya *et al.*, 2004). In this study using PLZF and VASA double fluorescent staining increased the accuracy of the ovine germ cells

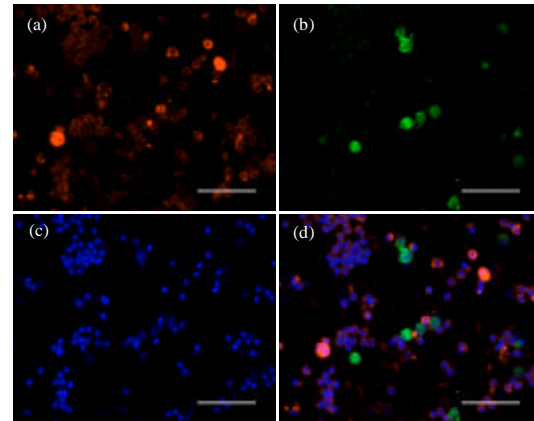


Fig. 3: Fluorescence double staining of sheep isolated testis cell for VASA and Vimentin; a) Red indicates Vimentin-positive cells; b) Green indicates VASA-positive cells; c) Blue indicate DAPI staining of the nuclei and d) Merged picture of the VASA and Vimentin. Scale bar = 50  $\mu$ m

identification. The expression of PLZF in testis stem cells decreased during ovine testis development as observed the decreased staining intensity in the peripubertal, prepubertal and post-pubertal ram testes which indicated that neonatal ram testes were much more suitable for ovine SSCs isolation and enrichment. In the present study, PGP9.5 staining was cytoplasmic whereas Vimentin staining was predominantly perinuclear. Merged images from double staining demonstrated that PGP9.5-positive cell were negative for Vimentin. Isolation efficiency of the testis germ cell also show neonatal ram testis should be better for ovine SSCs isolation and enrichment.

#### CONCLUSION

In this study using PLZF and VASA double fluorescent staining increased the accuracy of the ovine germ cells identification. Results indicated that testes from neonatal rams had only one type of undifferentiated germ cell gonocyte and should be suitable for ovine SSCs isolation and enrichment.

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