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The Ubiquitin C-Terminal Hydrolase L-1 (PGP9.5) as a Suitable Marker to Trace and Isolate Spermatogonia Cells from Ovine Testis

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Abstract: The Ubiquitin C-terminal Hydrolase L-1 (UCH-L1) is usually named as PGP9.5. Earlier study indicated that PGP9.5 has been used to identify type A spermatogonia in some species such as mouse and pig. In the present study, the localization of PGP9.5 was studied on paraffin sections of the ram testis samples in order to understand the correlation between gonocyte migration and spermatogonia location in the seminiferous tubules of ovine testis development. The results also indicated that testes from neonatal ovine had only one type of undifferentiated germ cell gonocyte and PGP9.5 as a spermatogonia cell marker was suitable for isolation and enrichment of ovine Spermatogonial Stem Cells (SSCs). Thus, the finding will be helpful for the future study on ovine SSCs in long-term culture and transplantation.

Key words: Ovine, spermatogonia, PGP9.5, immunohistochemical staining, SSC, gonocytes

INTRODUCTION

In order to identify and study the SSCs in testis all types of spermatogonia cell populations should be first characterized. Up to now, several spermatogonia cell populations in testis have been defined by using surface markers in earlier investigations. Application of c-kit as a marker for isolation of spermatogonia resulted in the selection of more differentiated spermatogonia than spermatogonial stem cells (Shinohara and Brinster, 2000). Isolation of mouse spermatogonia was on the basis of α-6 and β-1 integrin as markers which resulted in an enriched population of spermatogonial stem cell (Shinohara *et al*., 1999). Known as type A spermatogonia marker for bovine, the Dolichos Biflorus Agglutinin (DBA) was not to bind to any type of cell in prepubertal ovine testis (Rodriguez-Sosa *et al*., 2006).

The Ubiquitin C-terminal Hydrolase L-1 (UCH-L1) also named as PGP9.5 was earlier found with the high and specific expression in the mouse testis/ovary and neuronal cells (Kon *et al*., 1999). Afterward, PGP9.5 has been used to identify type A spermatogonia in several species including mouse (Kon *et al*., 1999) bovine (Zhang *et al*., 2008), human (Von Kopylow *et al*., 2010), porcine (Zeng *et al*., 2009) and ram spermatogonia (Rodriguez-Sosa *et al*., 2006; Herrid *et al*., 2009; Borjigin *et al*., 2010).

The aims of the present study were to confirm the extent of PGP9.5 during the specific period of ovine testis development. Subsequently, the PGP9.5 positive cells was specifically determined for their co-localization of PGP9.5 with the beta-Actin by using antibody double staining. After double antibody staining, PGP9.5 and vimentin as markers were simultaneously used to evaluate the efficiency for isolation and enrichment of spermatogonia cells from ovine testis.

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.

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Collection of testis samples: Rams were castrated under general anaesthesia (0.1 mg kg^{-1} xylazine followed by 3 mg kg^{-1} 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.

Immunocytochemistry staining of testis tissues with PGP9.5: Testis studies were rehydrated through xylene and ethanol then into water. Antigen retrieval was performed on slides by heating in 0.01 M citrate buffer (pH 6) for 10 min on the high setting in a microwave and then cooling for 30 min. Slides were then permeabilised for 5 min in Tris-Buffered Saline (TBS) with 0.01% Triton X-100 (Sigma-Aldrich) and then rinsed twice in TBS, pH 7.5. Sections were treated with 0.6% (v/v) H_2O_2 (Merck, Darmstadt, Germany) for 10 min to inhibit endogenous peroxidase and were subsequently rinsed in TBS. In order to block non-specific adhesion sites, slides were incubated in TBS containing 0.05% Tween 20 (TBST) for 30 min before antibody incubation. Slides were then incubated with rabbit anti-PGP9.5 (1:800; Dako, Glostrup, Denmark) for 30 min. Slides were then rinsed thoroughly three times with TBST for 5 min each time and incubated with En Vision (DakoCytomation; Dako) for 30 min at room temperature. Specific staining was visualized using diaminobenzidine kits (Vector Laboratories, Burlingame, CA, USA). Rabbit immunoglobins (Sigma-Aldrich) were used as negative controls for primary antibodies with replacement of the secondary antibody with buffer regarded as a further negative control. All incubations were performed in a moist chamber.

Double fluorescent staining of testis tissues with PGP9.5 and beta-actin: 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-beta-Actin (1:100) and rabbit anti-PGP9.5 (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^{-1}; 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^{-1}; Gibco-BRL) in PBS for 5-10 min at 37°C. DNAse I (7 mg mL^{-1}; 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 though 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 40-80×0^6 cells mL^{-1}. Cell viability was assessed by Trypan blue exclusion.

Enrichment of spermatogonia by differential plating: For differential plating, cell culture flasks (125 cm^2; Nunc, Roskilde, Denmark) were treated with 0.2% gelatin in PBS for at least 1 h at 37°C. Prior to the addition of testis cells, the PBS was replaced by 20 mL DMEM+5% FCS with antibiotics. About 50 million cells were added per flask, resulting in a final density of $0.4\times10^6/cm^2$. Following 2 h incubation at 37°C, the supernatants were poured off into 50 mL Falcon tubes followed by gentle rinsing twice with 5 mL PBS to harvest any loosely bound cells. The cell suspension was centrifuged at 400 g for 5 min at room temperature and the pellets from three or four Falcon tubes were combined and resuspended in 10 mL DMEM/F12+5% FBS. About 30 million cells were added to a new cell culture flasks (75 cm^2; Nunc) that were treated with 0.2% gelatin in PBS for at least 1 h at 37°C and then incubated overnight in DMEM + 5% (16 h). Thus, the final incubation density was $0.4\times10^6/cm^2$. The collection process was the same for the 2 h enrichment. Each treatment was duplicated.

Double fluorescent staining of the isolated and enriched 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-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).

RESULTS AND DISCUSSION

The PGP9.5-positive cells in ram testis from ovine testis developmental stage 1 to stage 4: Micrographs of immunostained testis sections of each stage were shown in Fig. 1. As testis development progressed from gonocytes to spermatogonia, PGP9.5-positive cells migrated from a predominantly central to a basal position. At stage 1 (Fig. 1a), PGP9.5-positive cells were randomly distributed in the central area of the seminiferous tubule. After this stage, more than three aligned cells were observed from one tubule cross section. At stage 2, about 80% of the cross-section at least had four PGP9.5-positive cells (Fig. 1b). In all four stages, two or three aligned PGP9.5-positive cells could be easily observed (Fig. 1a-d).

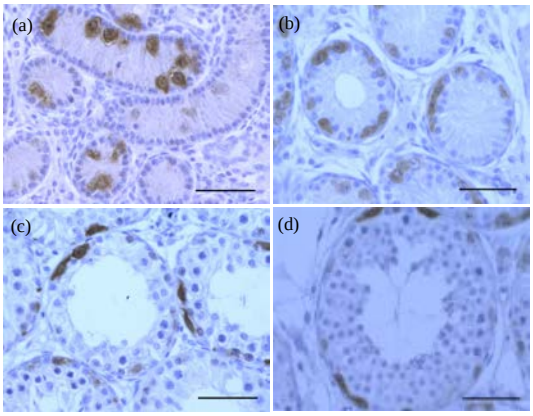

Fig. 1: Anti-PGP9.5 immuno-staining of ram testis sections from ovine testis four developmental stage. PGP9.5-positive cells observed here was consistent with gonocyte migration and spermatogonia location in the seminiferous tubules of neonatal; a) peripubertal; b) prepubertal; c) and post-pubertal d) ovine testes. Scale bars: 50 μm

The co-localization of PGP9.5 and beta-actin in different types of cell populations in ovine testis: Antibodies against PGP9.5 were earlier known capable of labeling spermatogonia cells (Wrobel, 2000). In the experiment of double antibody staining of testis samples on tissue sections with PGP9.5 and beta-Actin, researchers found that PGP9.5 positive cells were very weak stained for beta-Actin expression. However, the sertoli cells, myoid cells, differentiated spermatogonia cells and spermatocytes were all stained for the strong expression of beta-Actin. Remarkablly, PGP9.5-positive cells were found with the characters of round or oval shape in morphology (Fig. 2a-d).

The efficiencies for both isolation and enrichment of the neonatal ram testis germ cells by using PGP9.5 as a marker: Because of the fact that few spermatogonia cells exist in the seminiferous tubules to obtain sufficient cells used for SSCs long-term culture was regarded as one of difficulties in the present investigation. Presently, a successful method to obtain a significant amount of germ cells from the developing testis tissues at as early as stage 1 was expected to supply the pure and sufficient gonocytes or SSCs in study. The isolation method of gonocytes or SSCs could greatly promote the study on

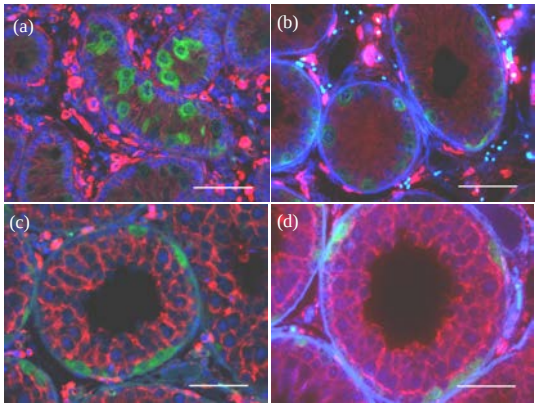

Fig. 2: Anti-PGP9.5 and Anti-beta-Actin immuno fluorescence staining of sheep testis sections at a) stage 1, b) stage 2, c) stage 3 and d) stage 4 of testis development showed that PGP9.5 positive cells were very weak stained for beta-Actin. PGP9.5 positive cells were dyed green and beta-Actin positive cells were dyed red. The nuclei were dyed blue using 4'-6'-Diamidino-2-Phenylindole (DAPI) staining. Scale bars: 50 μm

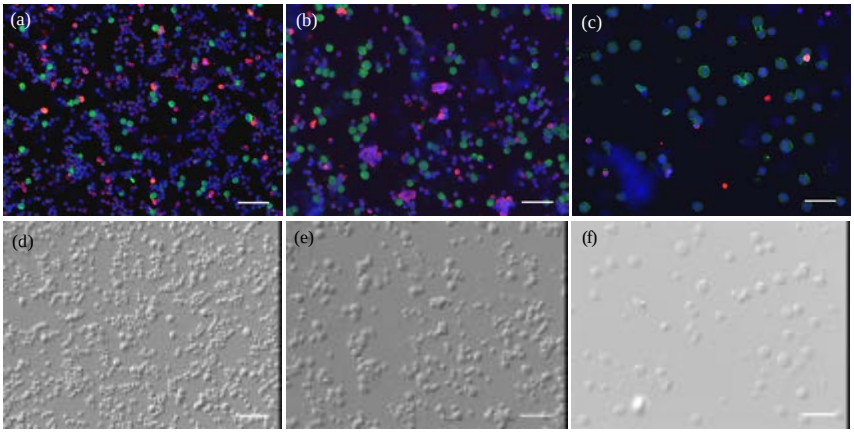

Fig. 3: Anti-PGP9.5 and anti-vimentin immuno fluorescence staining of isolated and enriched sheep testis cells. PGP9.5 positive cells were dyed green and Vimentin positive cells were dyed red. The nuclei were dyed blue using DAPI staining; a): Initial isolated cells; b): Enriched cells at 2 h later; c): Enriched cells at 18 h later; d, e and f were phase contrast micrographs of the stained cells in a, b and c respectively. Scale bars: 50 μm

SSCs in researches of culture and germ cell transplantation. In the experiment, two-step enzymatic isolation and differential plating procedure was established to obtain individual testicular cells and enrich gonocytes. The results of isolation and enrichment showed that most PGP9.5 positive cells in the tubular sections were recovered in the cell suspensions from stage 1 testis and showed good enrichment (Fig. 3).

Overall, the results indicated that the distribution pattern of the PGP9.5-positive cells observed here was consistent with gonocyte migration and spermatogonia location in the seminiferous tubules of neonatal, peripubertal, prepubertal and post-pubertal ovine testes. The results also indicated that the findings in ram testis for spermatogonia cell staining were similar to those previous findings of PGP9.5 expression pattern in bovine testis (Herrid *et al*., 2007). Remarkably, using both PGP9.5 and Vimentin or beta-Actin in double fluorescent antibody stainings increased the accuracy in identification of the ovine spermatogonia cells. In addition, the finding showed that the expression of PGP9.5 in testis cells decreased during ovine testis development. Researchers also observed the decreased intensity of PGP9.5 staining in the peripubertal, prepubertal and post-pubertal ram testes. Therefore, the results indicated that neonatal ram testes were likely more suitable for ovine SSCs isolation and enrichment.

On the other hand, researchers found that the enriched ovine testis cells were positive for PGP9.5 in cytoplasm whereas vimentin staining was predominantly in perinuclear. Merged images obtained from double antibody staining demonstrated that PGP9.5-positive cells were all negative for vimentin. These results suggested that the isolation and enrichment of ovine spermatogonia cells could be relatively easy for neonatal ram testis in comparison with peripubertal, prepubertal and post-pubertal ram testis.

CONCLUSION

The PGP9.5-positive germ cells of ram testis observed in the study is consistent with ram gonocyte migration and spermatogonia location in the seminiferous tubules of neonatal, peripubertal, prepubertal and post-pubertal ovine testes. The established method of successful isolation and enrichment of ram spermatogonia cells using PGP9.5 as cell surface marker will enable us to obtained sufficient SSCs from neonatal ovine testis during differential plating. Thus, the findings will be useful for obtaining sufficient cells in the investigation on the development mechanism of ovine testis germ cells. This method with two-step enzymatic isolation and enrichment of spermatognia cells will be applied for the ovine SSCs long-term culture and germ cell transplantation.

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