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Localization and characterization of SSCs from pre-pubertal bovine testes

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ABSTRACT

Self renewal and proliferation ability of spermatogonial stem cells (SSCs) support spermatogenesis during adult life. Theoretically, these stem cells can be utilized for transmission of genetic information to descendants via testicular transplantation. However, lack of knowledge in methodologies for identification of SSCs limits the application of SSCs transplantation in domestic animals. Accumulated studies have shown that SSCs specific markers (DBA, UCHL1) and stem cell marker (Sox2, Oct4) are useful to screen SSCs that able to be used for transplantation. However, in cattle, less information is available on the expression status of these markers till date. Therefore, a study was carried out in 2019 at Tsukuba University, Japan where testes from 3, 5 and 7 months old calves were utilized to examine testicular localization and *in vitro* propogation of stem cell markers. SSCs were isolated by enzymatic digestion combined with centrifugal separation on discontinuous Percoll density gradient. Cell propagation and SSCs marker expression were determined at 5, 10 and 15 days post-culture. Immunostaining in conjunction with Western Blot analysis of cultured cells showed that stem cell markers (UCHL1, Oct4 and Sox2) were expressed in SSCs suggesting that differentiation of gonocyte started by 3 months and SSCs differentiation begins after 5 months of age. Taken together, these results demonstrated marker expression and localization of bull SSCs and showed that *in vitro* culturing of bull SSCs is implementable.

Keywords: Bovine, Characterization, Localization, Pre-pubertal, SSCs, Testis

The feature of SSCs of being multipotent cells *in vitro* without any genetic manipulation, have attracted attention in several different fields like regenerative medicine and assisted reproductive technology. Shortly after birth, gonocyte enter the phase of cell division and gives rise to type A Spermatogonia, considered as the first generation of spermatogonial stem cells (Aponte *et al*. 2005). Specific germ cell markers have been expressed in different species. One such is DBA used for classifying and identifying spermatogonia from neonatal calves' testes (Herrid *et al.* 2007) and also expressed by bull gonocytes (Fujihara *et al.* 2011). Beside this, expression of transcription factors Sox2 and Oct4, which are critical for maintaining the pluripotency of stem cells (Shi and Jin 2010) and the transplantation assay (Brinster and Zimmermann 1994) are the other ways to determine characteristics of SSCs.

Functional proliferation of long term cultured SSCs in infertile recipient testis has been reported in rodents (Kanatsu-Shinohara *et al*. 2003) and porcine (Kim *et al*. 2008). However, in bovine, attemps to characterize SSCs by transplantation have not been successful (Izadyar *et al*. 2002). Further, the previously reported attempts to culture male germ cells for long term in cattle have not been successful (Izadyar *et al*. 2003, Oatley *et al*. 2004).

We aimed to understand physiological and biochemical characters of bovine SSCs, to build a practical system to generate progeny of SSCs by investigating expression of stem cell markers in *in vitro* cultured SSCs. To this end, we examined expression of different markers specific for stem cells, further we cultured SSCs from prepubertal testis. SSCs were evaluated by expression of markers and ability of proliferation after culture.

MATERIALS AND METHODS

Sample collection: Testes from 3, 5 and 7 months old calves (3 animals in each age category) were utilized for this experiment. The testes were collected in the Agriculture and Forestry Research Center at University of Tsukuba, Japan and National Institute of Livestock and Grassland Science, Tsukuba, Japan. All animal work was performed under the approval of the Institutional Animal Care and Use Committee of the University of Tsukuba (approval no. 18-397). Immediately after castration, the testicles were transferred to RNase free 10% PBS (pH 7.4), and transported to the laboratory within 1–2 h, in order to study the development of spermatogenic cells. Approximately 20 g of the testicular tissue was used for each cell isolation process. Subset of the sample were taken

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for immunohistological investigation followed by fixation with Bouin's solution.

Isolation and culture of germ cells: Individual tubular cells were isolated using a two-step enzymatic isolation process as previously described (Herrid *et al*. 2007) with minor modifications. Soon after removing testes capsule, 20 g of testicular tissues were minced in DMEM-F12 supplemented with 10% FBS, 50 IU/ml penicillin-streptomycine, 40 IU/ml gentamycin, 1.5 mg/ml collagenase 4, 2 mg/ml hyaluronidase type 2, 1.5 mg/ml trypsin and 30 µg/ml DNase 1. For semeniferous epithelial dispersion, the suspention containing minced testicular tissues were enzymatically digested at 37°C for 60 min in a shaking waterbath operated at 122 cycles/min. After centrifugation at 80 × g for 5 min, and removal of most of the interstitial cells, seminiferous cord fragments were re-suspended in 20 ml DMEM-F12 containing collagenase, hyaluronidase and DNase and incubated at 37°C for 45 min as above. Germ cells were separated from the remaining tubule fragments by centrifugation at 30 × g for 2 min followed by filtration through 70 µm nylon cell strainer. Then the suspension containing germ cells was centrifuged at 1500 × g for 5 min to pellet the germ cells. Cells were resuspended in 4 ml DMEM containing 10% FBS and 50 IU/ml penicillin–streptomycine and 40 IU/ml gentamycin. For further purificaiton, germ cells were subjected to discontinous percoll grandient layer (Izadyar *et al*. 2002) and the viability of cells was determined using live/dead staining. The identity of type A spermatogonia during isolation and purification was determined under a light microscope equipped with a Nomarski lens. Isolated cells were characterized further by using specific markers for type A spermatogonia, including Dolichos biflorus agglutinin (DBA). Cell purification was assessed by immunocytochemistry for SSCs markers and were incubated for 15 min with DBA-biotin (1:200) and UCHL1 antibody (1:300) diluted in PBS. Positive cells were counted under fluorescent microscope. About 1×10^5 cells/ml were seeded in DMEM-F12 supplemented with 10% FBS (v/v), 10 ng/ml EGF, 40 IU/ml gentamicin and 50 IU/ml penicillin – streptomycin and the medium was changed every two days.

Localization of germ cells in seminiferous tubules: SSCs were identified by evaluation under fluorescence microscope using DBA immunohistochemistry. Paraffin blocks of testis tissue were sectioned, deparaffinized, and antigen retrieval was performed as stated previously by Herrid *et al*. (2007). Slides were twice rinsed in PBS, incubated for 1 h at room temperature (RT) in 10% goat serum (v/v made in PBS) to block non–specific binding sites, consequently rinsed in PBS. The slides were then incubated in DBA biotin 1:200 (v/v) at 4°C overnight in moist chamber. After overnight incubation in DBA, the sections were rinsed 3 times in PBS. Staining of Fluorecence FITC – conjugated was performed by treating sections for 90 min in FITC (1:500 in PBS). The slides were then rinsed in PBS and nucleus was stained with DAPI. After overnight incubation, the slides were analyzed under fluorescence microscope. Further, the slides were double stained for Oct4, UCHL1 and Sox2 as described for cultured cells.

Characterization of cultured SSCs: Cultured spermatogonial stem cells were cytochemically analysed after 5, 10 and 15 days of culture as previously used by Fujihara *et al*. (2011) in large domestic animals, the physiological and biochemical characteristics of germ cells during the developmental processes remain largely unknown. In this study, we characterized bovine germ cells in the developing testis from the neonatal stage to the adult stage. The binding of the lectin Dolichos biflorus agglutinin (DBA) with modification. Briefly, cultured spermatogonial stem cells were cytochemically analysed after 5, 10 and 15 days of culture. The spermatogonial stem cells were stained with biotin DBA, Oct4, UCHL1 and Sox2. After washing two times with PBS, cells were fixed in 4% paraformaldehyde and incubated for 30 min at room temperature. Cells were washed with PBS twice, incubated in 10% (v/v) goat serum made in PBS for 60 min at room temperature, consequently washed twice with PBS and incubated for 90 min with primary antibodies viz. DBA-biotin (conjugate biotinylated, vectorlab; B-1035-5), UCHL1 antibody (Mouse Monoclonal; Am1959B Abgent), Oct4 antibody (AB3209 Millipore Sigma) and Sox2 (D6D9, Rabbit mAb, Cell Signaling) with concentrations of 1:200, 1:300, 1:200 and 1:400 (v/v) respectively diluted in PBS, followed by storage at 4°C overnight. After washing twice with PBS, cells were incubated with corresponding secondary antibodies viz. Neutra Avidin Fluorecence FITC – conjugated (1:500), fluorescence goat anti mouse IgG (173667) (1:300) and Texas red anti mouse IgG (H+L) (vector, made in horse 1:300) for 90 min at room temperature. Then the slides were washed again with PBS, incubated in DAPI nuclear staining and the cells were analyzed under fluorescence microscope.

Western blot analysis of cultured SSCs: Cells attached to the surface of culture wells were collected, washed with PBS and were supernatant, the sample was diluted with 10% Protease inhibitor EDTA free solution (made in PBS), followed by homogenization. Immediately, 10% Triton X – 100 (0.5% of the initial volume of sample) was added and mixed well. Later, cells were sonicated around 20 times (1 – 2 sec each), protein quantity was measured and Western blot was performed as described previously (Priyadarshana *et al*. 2018). The membrane was incubated overnight at 4°C with respective primary antibody, UCHL1, Anti-Oct4 and Sox2. All were diluted in TBS (1:1500 v/v). Following 3 washes, those were incubated with respective anti-mouse and anti-rabbit HRP conjugated secondary antibodies (1:10,000 dilution) for 1 h at room temperature and immunoreactivity was detected with Molecular Imager Chemi Doc ™ XRS + with Image lab™ (Bio – Rad Laboratories, Inc. USA).

Statistical analysis: The results are presented as mean±SEM and statistical analysis was peformed by one

way ANOVA followed by Tukey HSD Test for multiple comparisons. Differences were considered significant when the P value was <0.05.

RESULTS AND DISCUSSION

To localize spermatogonial stem cells, DBA biotin staining was performed (Fig. 1). In 3 months old testes,

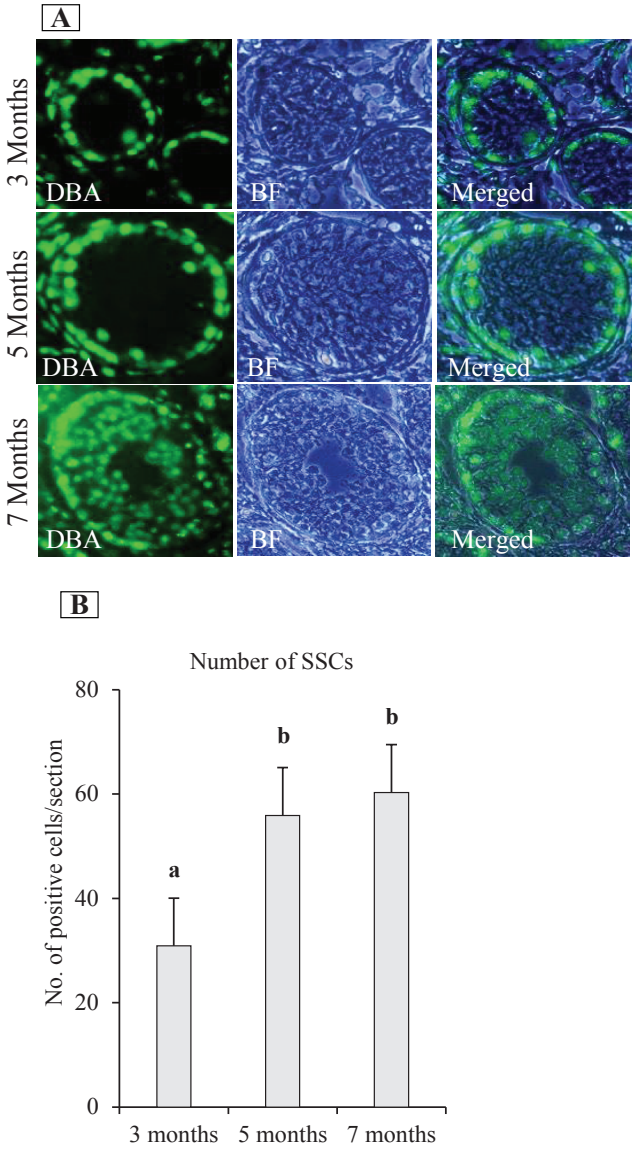

Fig. 1. Histological sections of bovine testes at 3, 5 and 7 months of age were immunolocalized with DBA, a specific marker for SSCs. Images are representative of three independent experiments. **(A)** Number of DBA positive cells were counted. **(B)** Data are presented as mean±SEM (n=10). $^{a\text{-}b}P<0.05$.

few DBA positive cells were localized in the middle of tubes having large size, round shape and one spherical nucleus stationed at the center of cell. While in the basement membrane, the number was high, the cells were small in size and had 1 or 2 unorganized nuclei. This observation is consistent with evidences that cells in the middle proportion were gonocytes (Fujihara *et al*. 2011) and cells located in the basement membrane were type A spermatogonia (Zhao *et al*. 2016). In 5 months old testis, DBA positive cells were located both in basement membrane and just next to the basement membrane. The cells were large in size, round shape and had dense nucleus. Therefore, in bovine testis, differentiation of gonocyte to spermatogonial stem cells may begin at 3 months of age, but may not be completed until 6 months of age (Herrid *et al*. 2007). Likely, in 7 months, positive cells were located in both topographical locations. The number of cells in basal membrane was not significantly different than 5 months but was significantly different for cells in the middle portion. Difference in the cell size shows different phases of cell cycle (Lok *et al*. 1982), whereas, 3 generations of different size type A spermatogonia basal stem cells, aggregated spermatogonia, and differentiating spermatogonia have been shown by electron microscopy (Wrobel 2000). In this way, cells next to the basement membrane might be A1 - A4 spermatogonia.

Further, we double stained the cells with UCHL1, Oct4, and Sox2. Staining showed, UCHL1 expression was restricted to cells in the basement membrane. However, some cells were positive for UCHL1 but were not positive for DBA, but the number was not significantly different. Equal number of positive cells were located in basement membrane and middle portion for DBA and UCHL1 in 7-months old testes, unlike in 5 months where there was no positive cells for UCHL1 (Fig. 2). UCHL1 expression is restricted to spermatogonial stem cells of prepubertal bull (Herrid *et al*. 2007, Reding *et al*. 2010), cat (Vansandt 2014), prepubertal buffalo (Goel *et al*. 2010) and porcine (Zhao *et al*. 2016). However, in neonatal and adult bovine testis, UCHL1 is expressed in gonocytes and spermatogonia (Fujihara *et al*. 2011). In this regard, our results suggest that in 3 months old cells in the middle portion is gonocytes and in 7 months, the stained cells might be result of non-specific binding.

Cells in the basement membrane from the above ages were also positive for Sox2 (Fig. 3). No Oct4 positive

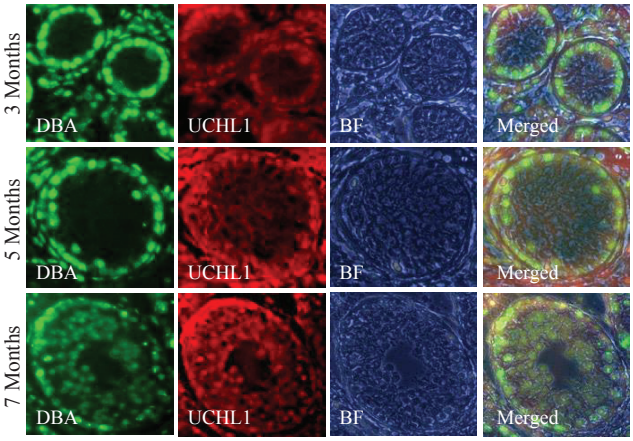

Fig. 2. Histological sections of bovine testes at 3, 5 and 7 months of age were co-immunolocalized with DBA and UCHL1, specific markers for SSCs. Images are representative of three independent experiments.

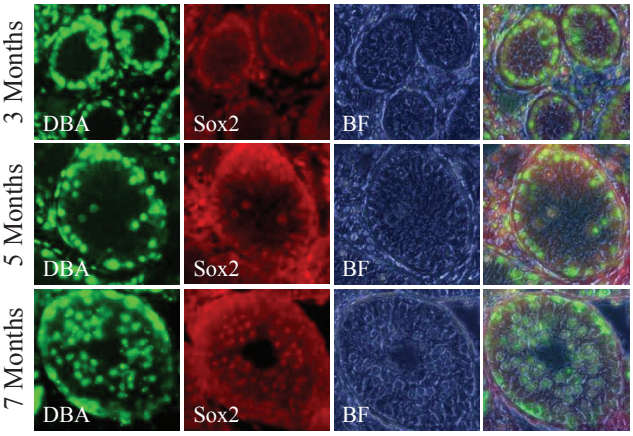

Fig. 3. Histological sections of bovine testes at 3, 5 and 7 months of age were co-immunolocalized with DBA and Sox2. Images are representative of three independent experiments.

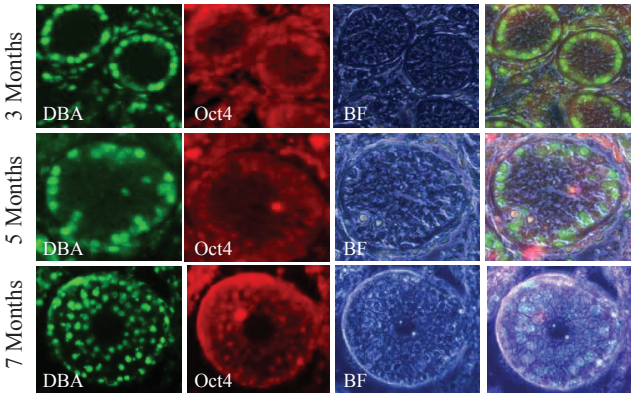

Fig. 4. Histological Sections of bovine testes at 3, 5 and 7 months of age were co-immunolocalized with DBA and Oct4. Images are representative of three independent experiments.

cells were detected in middle part of 3 and 5 months old seminiferous tubes. In 7 months old, cells next to basement membrane were positive for Oct4 (Fig. 4). Oct4 and Sox2 are transcriptional factors critical for maintaining and inducing pluripotency of stem cells (Oatley *et al*. 2006, Zhang and Cui 2014). Our results showed that these factors could be detected in spermatogonial stem cells of prepubertal bovine. Like other SSCs markers, Oct4 is used to mark spermatogonia at different ages (Fujihara *et al*. 2011) including stem spermatogonia (Manku and Culty 2015). Expression of Oct4 is reported in single (As), paired (Apr) and aligned (Aal) spermatogonia of cattle (Aponte *et al*. 2008), gonocyte and spermatogonia of buffalo (Mahla *et al*. 2012). In addition, Oct4 induces totipotency and its expression is also reported in interstial cells and differentiated spermatogonia (Goel *et al*. 2008).

Bovine SSCs from 3, 5 and 7 month old calves were cultured *in vitro*. After 5 days of culture, the cells proliferated and adhered to the surface of culture well. About 80% of surface was covered and SSCs represented colony

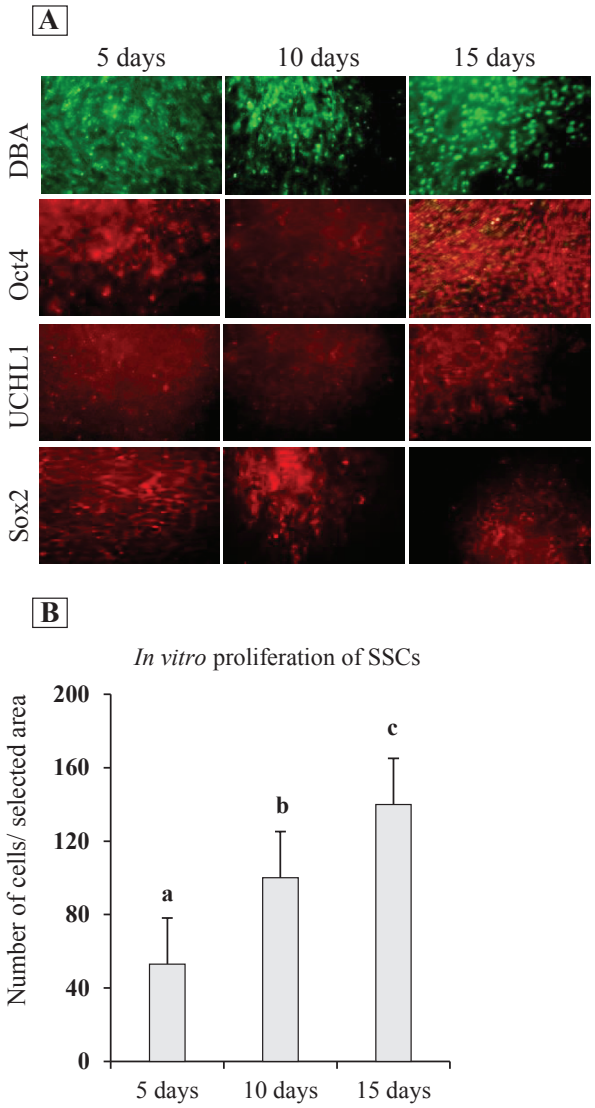

Fig. 5. Bovine SSCs were cultured and stained with stem cell markers, viz. DBA, Oct4, UCHL1 and Sox2. Expression of markers were examined at 5, 10 and 15 days post *in vitro* culture. Images are representative of three independent experiments. **A**. Number of DBA positive cells at 5, 10 and 15 days post *in-vitro* culture were counted. **B**. Data are presented as mean±SEM (n=10). $^{a\text{-}c}P<0.05$.

like structure with 2 different morphologies viz. three dimensional cell mass and scattered form. To characterize these colonies and cells, they were immunocytochemically analyzed after 5, 10 and 15 days of culture, showing that they were DBA positive. Further, UCHL1, Sox2 and Oct4 were also positive. Besides, when double staining was performed for the presence of DBA and stem cell markers, some DBA positive colonies were devoid of stem cell markers and conversely, some positive cells for stem cell markers were devoid of DBA. The number of colonies almost doubled from 5 (110±0.57) to 10 days (192±1.43) and then further increased by 15 days (210.5±1.21). At 10 days of culture, the colonies had spherical shape, but was changed to long shape at 15 days of culture (Fig. 5). Study of cultured cells revealed that 5 days old cultured

SSCs are able to make colonies, and the number of colonies increases further by extended culture period until 15 days. Furthermore, the shape of colonies after 15 days of culture was elongated, which might be resulted from effect of EGF (Aponte *et al.* 2008).

The colonies were consistently positive for the markers. Supporting this, Western blotting showed expression of UCHL1, Oct4 and Sox2 related protein to be present in cultured SSCs (Fig. 6). Oct4 is member of pluripotency regulating protein network (Manku and Culty 2015), together with Sox2, these factors promote stemness of ES cells (Oatley and Brinster 2008), and pluripotency of adult fibroblast cells in bovine (Pan and Ebner 2014). Similarly,

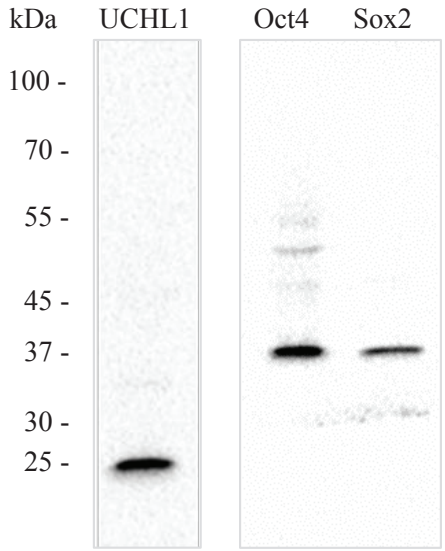

Fig. 6. Cultured SSCs were subjected to immunoblotting with antibodies against UCHL1, Oct4 and Sox2. UCHL1, Oct4 and Sox2 protein were expressed at the predicted molecular weight. Images are representative of three independent experiments.

the expression of Sox2 and Oct4 is not restricted to specific type of germ cells. For instance, Oct4 expression have been seen in differentiated types of spermatogonia in pig but rarely in SSCs (Goel *et al.* 2008). Furthermore, presence of Oct4 and Sox2 in mitotic chromatin is reported to be crucial for pluripotency maintenance (Deluz *et al.* 2016). Whereas Sox2 mechanism is specific to cell type (Hagey *et al.* 2018). In addition to this, Oct4 has two different activities, inducing pluripotency and committing cell differentiation (Stefanovic *et al.* 2009), and Sox2 is required for directing pluripotent stem cell's differentiation (Zhang and Cui 2014). In line with this, Oct4 and Sox2 are able to promote cell differentiation fate (Zeineddine *et al.* 2014).

Putting all these together, our results indicated that, cells in the basement membrane were spermatogonia and that after isolation and culture, colonies continuously increased. On the other hand, the cells in the adluminal compartment were differentiated type germ cells that can be identified based on their geographical location and morphology.

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