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Comparing the stemness and morphobiometry of spermatogonial stem cells from Doom pig on different days of culture

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Abstract: The present study was conducted to compare the stemness and morphobiometry of spermatogonial stem cells (SSCs) from the Doom pig on different days of culture (9th, 30th and 65th day) for the development of long-term culture method. The testes from 7–15-day old piglets were collected and two-step enzymatic digestion was used to isolate SSCs. Before *in vitro* culture of SSC-like cells on the Sertoli cell feeder layer, the cells were enriched by differential plating and Percoll density gradient centrifugation. The isolated SSCs were characterised by alkaline phosphatase and immunofluorescence staining and qPCR analysis of SSC specific marker genes. Stemness was compared based on the expression of different SSC specific marker genes. The putative spermatogonial stem cells (PSSCs) from all the days of culture were found to be positive for alkaline phosphatase and immunofluorescence staining. The results from qPCR analysis showed that PSSCs were positive for SSC marker genes, though their expression decreased gradually from day 9 to day 65 of culture. The shape of the cells was found to change from compact round or oval to amorphous shape on day 65 of culture. Colony diameter ranged from 68.92 ± 1.20 µm (day 9) to 213.53 ± 12.52 µm (day 65) and differed significantly from each other. The number of colonies on day 65 of culture was significantly lower than on days 9 and 30. These results suggest that the enriched SSCs from Doom pigs can be maintained up to two months *in vitro* in the present culture system.

Keywords: porcine spermatogonial stem cell; long-term culture; immunofluorescence staining; qPCR; colony diameter; colony number

The spermatogonial stem cells (SSCs) are germ stem cells originated from seminiferous tubules in testes, and they produce sperm through the process of spermatogenesis. However, at the same time, they keep their cellular pool constant through self-renewal. SSCs are biotechnologically important because they are the only adult stem cells capable of transmitting genetic information to progeny generations. SSC biotechnologies are also aimed to solve fertility-related issues. SSC suspensions

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can be transplanted to the testis of an infertile animal and are promising for fertility restoration and preservation of endangered species. Bioengineered or gene-edited SSCs transplanted into testes generate transgenic sperm and could enhance the efficiency of transgenic animal production (Sun et al. 2019). Testis germ cell transplantation (TGCT) is emerging as a novel reproductive technology with application in animal breeding systems, including the potential for use as an alternative to artificial insemination, an alternative to transgenesis, part of an approach to reducing generation intervals, or an approach toward development of interspecies hybrids (Herrid and McFarlane 2013). SSCs also have the capacity to convert into pluripotent stem cells (Kanatsu et al. 2008). Therefore, SSCs are of particular utility in animal genetics, breeding, reproduction and conservation. It might have a much greater impact on pig breeding as the advantages from artificial insemination with frozen semen cannot be exploited to its fullest because of poor cryosurvival of the boar semen (Hernandez et al. 2007). However, to reach these goals using SSCs, a large number of SSCs must be maintained under *in vitro* culture conditions and it also requires species-specific knowledge of SSC culture. SSCs are localised on the basement membrane of seminiferous tubules in a specific microenvironment or niche. The niche comprises Sertoli cells, Leydig cells, myoid cells, a number of growth factors synthetised by Sertoli cells and other somatic cells, blood vessels and basement membrane (de Rooij 2009). Thus, the growth factors like glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor (FGF), epidermal growth factor (EGF) are essential for self-renewal and maintenance of SSCs during *in vitro* culture. However, long-term culture of SSCs remains challenging due to the difficulties in maintaining the SSCs in culture conditions that support their self-renewal, molecular and cellular characteristics and pluripotent potential (Kokkinaki et al. 2011). Although long-term culture of rodent SSCs has been developed and SSCs are able to proliferate for over five months, no efficient long-term culture method of SSCs in large animals, including boars, has been developed yet (Zhang et al. 2017). It seems that SSCs from large domestic animals possess some unique characteristics and the established culture systems developed for rodent SSCs could not support the long-term proliferation of SSCs from livestock species (Sahare et al. 2016). Luo et al. (2006) established a method of enriching spermatogonial cells from the pig testes using protein gene product 9.5 (*PGP9.5*) as a marker and could maintain the culture for two weeks without losing their *PGP9.5* expression. However, the first report on porcine SSCs probably appeared in 2006 wherein Cheng and Feng (2006) successfully isolated SSCs in minipigs. But it was not possible to maintain these minipig SSCs in culture for more than 10 days (Han et al. 2009). The lack of fitting feeder cells for subculture is also one of the bottlenecks for the development of the culture system. A previous study showed that undifferentiated spermatogonia could survive *in vitro* for one month when co-cultured with autologous Sertoli cells (Zheng et al. 2013).

A key step in studying the biology of SSCs during culture is to determine their gene expression profile (Kokkinaki et al. 2009). Differences in gene expression often correlate with differences in stemness (Mutoji et al. 2016). Over the past 10 years, a panel of markers for stemness has been assessed to characterise and identify human spermatogonial stem cells (Dym et al. 2009). *OCT4* and *SOX2* are two such transcription factors required for the maintenance of pluripotency of embryonic stem cells, also considered as stemness marker for SSCs (Kala et al. 2012; Hamidabadi and Bojnordi 2018). Though most of the works have been reported in mouse and human SSCs, in recent years some progress on the isolation, purification, and genetic manipulation of porcine SSCs has also been reported. However, the perusal of available literature reveals no work on porcine SSC culture in India.

Pig is considered as the most precious meat-producing animal in Assam and other northeastern parts of India. Almost all tribal people in the region are non-vegetarians and pork is their first choice of meat. However, per capita consumption of pork is very poor ranging from 0.069 to 0.532 kg per month and far below than recommended by the Indian Council of Medical Research (ICMR). The gap between production and requirements for pork is due to the poor production potentiality of indigenous pigs and lack of scientific breeding practices in the region. To meet the escalating demand for pork in the region, upgradation of indigenous pigs is of paramount importance. Doom is one of the indigenous pigs found in Assam that is black in colour with thick hair on the neck. This variety of

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pig is poor in productive and reproductive efficiency although it possesses valuable traits such as disease resistance, good maternal qualities, unique product quality, strong fitness under extensive low-input management conditions and adaptability to harsh environment and ability to thrive on poor quality feed. The number of Doom pigs is only 15 725 and also a negative growth rate (−18.22%) was registered from 2007 to 2012, which is much higher than the national average (19th Livestock Census, Government of India for 2012). Therefore, it is required to explore the newer areas that can find application in genetic improvement as well as more efficient conservation of these indigenous pigs which can ultimately uplift the economic status of poor tribal people of the areas. One such area could be male germline stem cell technology that still remains unexplored in this pig. Thus, the present study was conducted with the aims to compare the stemness and morphobiometry of SSCs from Doom pig on different days of culture to develop a long-term culture method.

MATERIAL AND METHODS

Animal care

The Institutional Animal Ethics Committee (IEAC) of the Faculty of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati-22, India has approved all the animal procedures related to the present study (770/ac/CPCSEA/FVSc/AAU/IAEC/17-18/615 dated 09/08/2017).

Collection of testis samples

Testes were collected from 7–15 days old prepubertal male Doom piglets and transported to the laboratory within 2 h from collection under cold (15–25 °C) and aseptic conditions. The testes were washed twice with 0.9% normal saline containing 400 IU/mL penicillin and 500 µg/mL streptomycin and subsequently visible connective tissues were removed with a sharp surgical blade. They were kept in 70% alcohol for 1 min and again washed 7–8 times with normal saline containing antibiotics [50 µg/mL Gentamycin (Sigma-Aldrich, USA, G1264) and 10 µL/mL penicillin-streptomycin (Gibco, USA, 15070-063), and Amphotericin solution (Sigma-Aldrich, A5955)].

Experimental procedure

The collected samples of the testes were subjected to double enzymatic digestion, differential plating and Percoll density gradient centrifugation methods for the isolation and enrichment of SSCs and co-cultured with the Sertoli cell feeder layer. Morphobiometry, i.e. morphology, diameter and number of SSC colonies was recorded on different days of culture. Alkaline phosphatase staining, immunofluorescence staining and the expression study of SSC specific pluripotent marker genes were done to characterise putative spermatogonial stem cells (PSSCs). Stemness of SSCs was compared based on the expression of SSC specific pluripotent marker genes.

Isolation and enrichment of SSCs

Isolation of spermatogonial stem cells was carried out as per the method described by Kala et al. 2012. Briefly, the testes were again washed with calcium and magnesium free Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich, D8537) containing antibiotics after brought to the actual place of work. Any visible extra connective tissues and *tunica albuginea* were removed and seminiferous tubules were dissociated and minced into small pieces in a Petri dish containing DPBS with antibiotics with the help of sterile scissors and forceps. About 4–5 g of minced tissues were taken and two-step enzymatic digestion was used to obtain single cell suspensions. Tissues were incubated in Dulbecco's Modified Eagle Medium (DMEM) containing 1 mg/mL of collagenase (Sigma-Aldrich, C-2674), 1 mg/mL of hyaluronidase type II (Sigma-Aldrich, H2126), 5 µg/mL of DNase I (Sigma-Aldrich, DN25) and 1 mg/mL of trypsin-EDTA (Sigma-Aldrich, T4049) for 45 min with shaking (145 cycles/min) at 37 °C. The digested tissue was collected and subjected to centrifugation at 1 000 rpm for 5 min and the supernatant was discarded. The tissue pellet was washed once in DMEM and a second digestion was given in the same digestion media for 30 min as described above. The activity of trypsin was stopped by addition of 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, F4135), 1% non-essential amino acid (NEAA) (Sigma-Aldrich, M7145) and 1% L-glutamine (Sigma-Aldrich, G5763) followed by centrifugation at 1 000 rpm for 5 min. The supernatant was collected in a 15 mL centrifuge tube which was again centrifuged at 1 000 rpm for 5 min to form the cell pellet. Thus, the obtained pellet was dissolved in DMEM after discarding the supernatant. This cell suspension was expected to contain SSCs, Sertoli cells, myeloid cells and

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other contaminating cells of the seminiferous tubular tissue and was filtered through 80 µm and then 60 µm nylon mesh filter (Millipore Corp., USA, NY8002500 and NY6002500) to enrich the putative SSC population. The filtered cells were further enriched through differential plating and Percoll density gradient centrifugation methods.

Differential plating. Differential plating was done to separate Sertoli cells from the isolated cell population according to Scarpino et al. 1998, with minor modifications. For this, bovine serum albumin-lectin (BSA-lectin) coated dishes were prepared by dissolving lectin from *Datura stramonium* (Sigma-Aldrich, L2766; 5 µg/mL) in DPBS and pouring 500 µL in each 35 mm Petri dish followed by 1 h incubation at 37 °C. The coated dishes were then rinsed three times with DPBS and again incubated with 0.6% of bovine serum albumin (BSA) (Sigma-Aldrich, A3059) in DPBS at 37 °C for another 1 h to allow the coating of BSA over lectin. The mixed population of the cells obtained through two-step digestion and filtration was transferred to the BSA-lectin coated dish and was incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Next day, after incubation, non-adhering cells obtained were subjected to Percoll density gradient cell separation for further enrichment.

Percoll density gradient centrifugation. An iso-osmotic Percoll suspension was prepared containing 82.2% Percoll (Sigma-Aldrich, P4937), 0.6% BSA and 45 µg/mL DNase I in DMEM. A discontinuous density gradient was prepared by diluting the iso-osmotic Percoll suspension with DMEM containing 0.7% BSA and 50 µg/mL DNase I. The gradients were created by layering 1 mL each of 65, 50, 40, 36, 34, 32, 30, 28 and 20% Percoll into a 15 mL centrifuge tube. The non-adhering cell pellet obtained after differential plating was suspended in 500 µL DMEM, 0.7% BSA and 50 µg/mL DNase I and was layered on top of the gradient. The gradient was centrifuged at 3 000 rpm for 30 min and 4 mL of Percoll was taken in a 15 mL centrifuge tube after removing 1 mL of Percoll from the top of the column as the second to the fifth layer of Percoll contains a maximum number of SSCs. Percoll was then removed and cells were washed by centrifugation and the pellet was dissolved in culture medium consisting of DMEM supplemented with 10% (v/v) FBS, 40 ng/mL glial cell line-derived neurotrophic factor (GDNF) (Sigma-Aldrich, G1401), 10 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich, E4127), 10 ng/mL fibroblast growth factor (FGF) (Sigma-Aldrich, F3133), 1% L-glutamine, 1% NEAA and it was cultured on the mitomycin-treated Sertoli cell feeder layer.

Preparation of the feeder layer

The cells which were expected to be primarily Sertoli cells left over in the lectin-coated dishes were rejuvenated with fresh DMEM supplemented with 10% (v/v) FBS and 1% NEAA and subsequently incubated in a CO_2 incubator at 37 °C for 3–4 days to allow these cells to grow to a confluent monolayer. The feeder cells were subcultured either in a 25 cm^2 culture flask or a 35 mm Petri dish by trypsinisation. Sertoli cells were allowed to grow to a monolayer of approximately 70% confluence and then were treated with 10 µg/mL mitomycin C (Sigma Aldrich, M0503) for 3 h, and after that they were washed 5 times with DPBS and finally with DMEM prior to the seeding of putative SSCs.

Culture of SSCs

The cells seeded onto the Sertoli cell feeder layer were cultured *in vitro* in the culture medium described above in an incubator with 5% CO_2 in air at 37 °C. The culture medium was replaced every fourth day and passaging was done on every 7^{th} day. Morphology, diameter and the number of colonies were regularly observed and recorded under an inverted fluorescence microscope Axio Vert.A1 FL-LED (Carl Zeiss Microscopy GmbH, Germany) and the data from days 9, 30 and 65 of culture were analysed using one-way ANOVA to compare the morphobiometry of SSCs.

Characterisation of SSCs

Characterisation of PSSCs was done by alkaline phosphatase staining, immunofluorescence staining and the expression study of SSC specific pluripotent marker genes on days 9, 30 and 65 of culture.

Alkaline phosphatase staining. The Alkaline phosphatase staining was done using an alkaline phosphatase staining kit (Sigma-Aldrich, 86C) following the manufacturer's instructions. Briefly, PSSCs were washed with PBS after fixing in a fixative solution and then incubated with alkaline dye for about 15 min. The cells were again washed with PBS several times after counterstaining and

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alkaline phosphatase activity was determined by visual analysis of the stained cells. Positive SSC colonies were visualised as red colour colonies under a microscope (Ju et al. 2008).

Immunofluorescence staining. The immunofluorescence staining of the SSC colonies was performed using primary antibodies [*OCT4* (Abcam, UK; ab181557), *ITGB1* (Invitrogen, USA; PA5-29606), *SSEA1* (Invitrogen, MA1-022) and *PGP9.5* (Abcam, ab10404)]. For Sertoli cells, the primary antibody, Vimentin (Invitrogen, MA5-11883) was used. Secondary antibodies [Sheep anti-Rabbit, FITC (Abcam, ab6791) for *OCT4* and *PGP9.5*; Goat anti-Mouse, Alexa Fluor 488 (Invitrogen, A-11001) for *SSEA1*; and Vimentin and Goat anti-Rabbit, Alexa Fluor 488 (Invitrogen, A-11034) for *ITGB1*] were used in the staining procedure. Hoechst (Sigma-Aldrich, H6024; 1 µg/mL) and propidium iodide (Sigma-Aldrich, P4170; 100 ng/mL) were used to stain the nucleus.

The cultured SSC cells in 96-well plates were washed 2–3 times in DPBS-polyvinyl alcohol (PVA) (Sigma-Aldrich, P8136) and allowed to fix in 4% paraformaldehyde (Sigma-Aldrich, P6148) for 30 min at room temperature. The fixative was aspirated out and rinsed thrice with DPBS-PVA before proceeding to immunostaining. After blocking with 2% BSA in PBS, 200 µL of primary antibody was added to the well and stored at 4 °C overnight. Next day, the cells were washed thrice with DPBS-PVA and then secondary antibody was added to the well and incubated for 1 h. Secondary antibody was removed and the cells were again washed with DPBS. Hoechst or propidium iodide was added and kept at room temperature for 1 min for counterstaining. After washing three times, the plate was examined under a fluorescence microscope (Beaulah et al. 2016). Negative controls without primary antibody were used to determine if the secondary antibody is binding nonspecifically to cellular components, resulting in false positives or nonspecific binding.

Quantitative real-time expression of SSC specific pluripotent marker genes

RNA isolation and cDNA amplification. The spermatogonial colonies were detached from the feeder layer on days 9, 30 and 65 of culture by trypsinisation to obtain a single cell suspension. Total RNA was then isolated using a PureLink RNA Mini Kit (Invitrogen, 12183018A) as per manufacturer's protocol. The cell lysate was used for cDNA synthesis by means of a high capacity cDNA kit (Invitrogen, 4368814).

SYBR Green based real-time PCR for quantification. The expression of SSC specific pluripotent marker genes *SOX2* and *OCT4* (Bai et al. 2016) was analysed in the present study. The purity of SSCs was also confirmed by the negative expression of *c-Kit* and *PPARγ*, which are the markers for differentiating spermatogonial and germ cells (Han et al. 2009). Quantitative real-time PCR was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, USA) in a 10 µL reaction volume containing 5 µL of SYBR Green Master Mix, 0.5 µL of each primer of 2.5 pmol concentration and cDNA. The thermal cycling conditions consisted of 1 cycle of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, followed by 1 cycle of melt curve stage at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. All primer pairs used were confirmed for their PCR efficiency and specific products were checked by the melt curve analysis. For primer sequences used for the genes see Supplementary Table S1 in Electronic Supplementary Material (ESM). *ACTB* was used as the housekeeping gene. A non-template control (NTC) was also taken for each gene with every batch of cDNA preparation.

The relative quantification of the target gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The threshold cycle (Ct) values were based on triplicate measurements. The data analysis was carried out by StepOne software Version 2.2.2. Data were analysed by one-way ANOVA, wherever required. Each PCR product was electrophoresed for visualisation of specific products.

RESULTS AND DISCUSSION

Isolation, enrichment and *in vitro* culture of spermatogonial stem cells and Sertoli cells

One of the crucial steps to obtain a sufficient number of viable SSCs is proper disaggregation of the testicular tissues which determines the efficiency of the SSCs isolation from the mammalian testis. Broadly, disaggregation is carried out either by a mechanical method or by a multi-step enzymatic digestion (Nasiri et al. 2012). Though the enzymatic digestion protocols are relatively complex and time-consuming, they yield good

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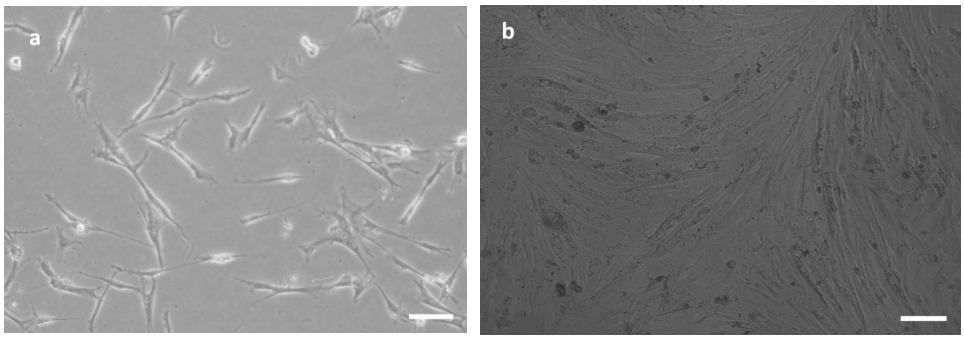

Figure 1. A Sertoli cell feeder layer from Doom pig on (a) day 3 and (b) day 9 of culture (scale bar: 100 μm)

results. In the present study, almost a pure population of PSSCs (as indicated by the positive alkaline phosphatase and immunofluorescence staining) was isolated by enzymatic digestion using four enzymes and different techniques of enrichment from the testes of Doom pig. The identity of PSSCs and Sertoli cells was first determined by analysing their morphology. After 3–4 days of culture, the adherent Sertoli cells showed a typical morphology with enlarged body and dramatically spread cytoplasm with 3–4 enations like an irregular polygon and formed a monolayer with approximately 70% confluence after 7–9 days (Figure 1). SSCs were observed as three-dimensional dome-shaped round or oval bodies that began to form clusters after 4 days and were visible on days 6–9 of culture as single, paired or clustered cells. Around the 18th day of culture, SSCs formed a mulberry shaped small colony with a distinct boundary from the feeder layer. The morphology of cells observed in the present study was similar to the morphology of porcine SSCs derived by other investigators (Han et al. 2009; Shi et al. 2015). It was seen that the morphology of cells could be maintained till day 60 and was found to be deteriorated and changed from compact round or oval shaped to amorphous on day 65 of culture (Figure 2). Germ cells from the prepubertal cat testes also remained viable in culture up to 43 days (Guaus et al. 2017). Bai et al. 2016 reported that the typical morphological characteristics of mouse spermatogonial stem cells were maintained under *in vitro* culture conditions for over five months.

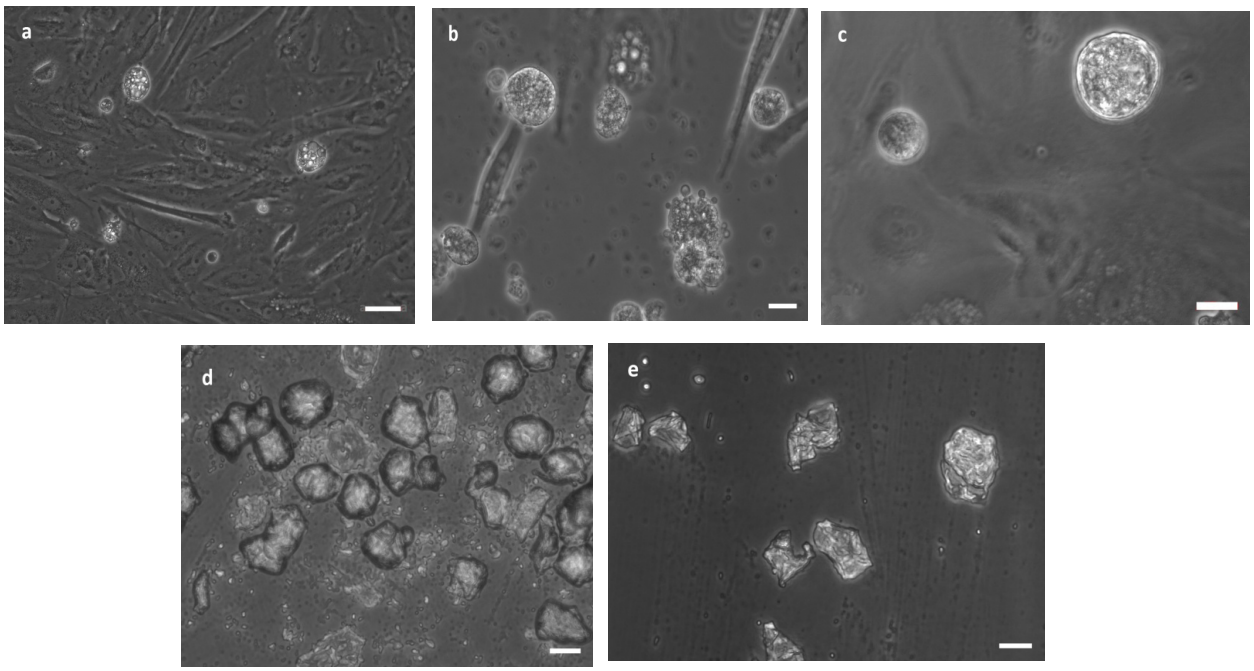

Figure 2. Morphology of spermatogonial stem cells (SSCs) from Doom pig on a Sertoli cell feeder layer. Formation of a dome-shaped SSC colony on (a) day 9 and (b) day 15 of culture; (c) a mulberry-shaped colony with a distinct boundary from the feeder layer on day 18; (d) on day 60; (e) morphology of the colonies gradually changed to amorphous on day 65 of culture (scale bar: 50 μm for a, d, e; 20 μm for b, c)

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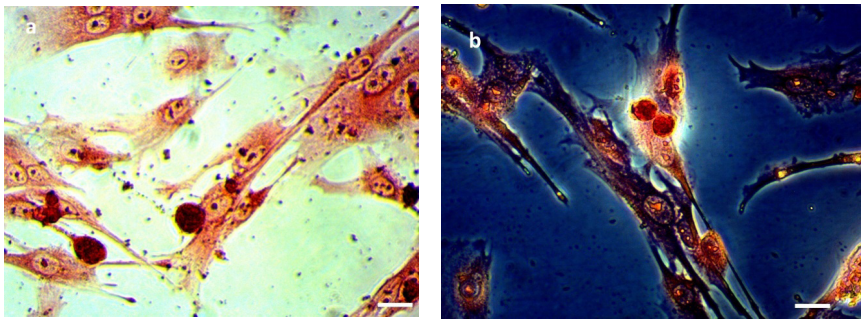

Figure 3. A spermatogonial stem cell colony on (a) day 9 and (b) day 30 of culture positive for alkaline phophatase staining (scale bar: 50 µm)

The diameter and number of colonies are presented in Supplementary Table S2 in ESM. The colony diameter significantly ($P < 0.01$) increased along with the day of culture and reached from 68.92 ± 1.20 µm on day 9 to 213.53 ± 12.52 µm on day 65 of culture. This is comparable with the findings

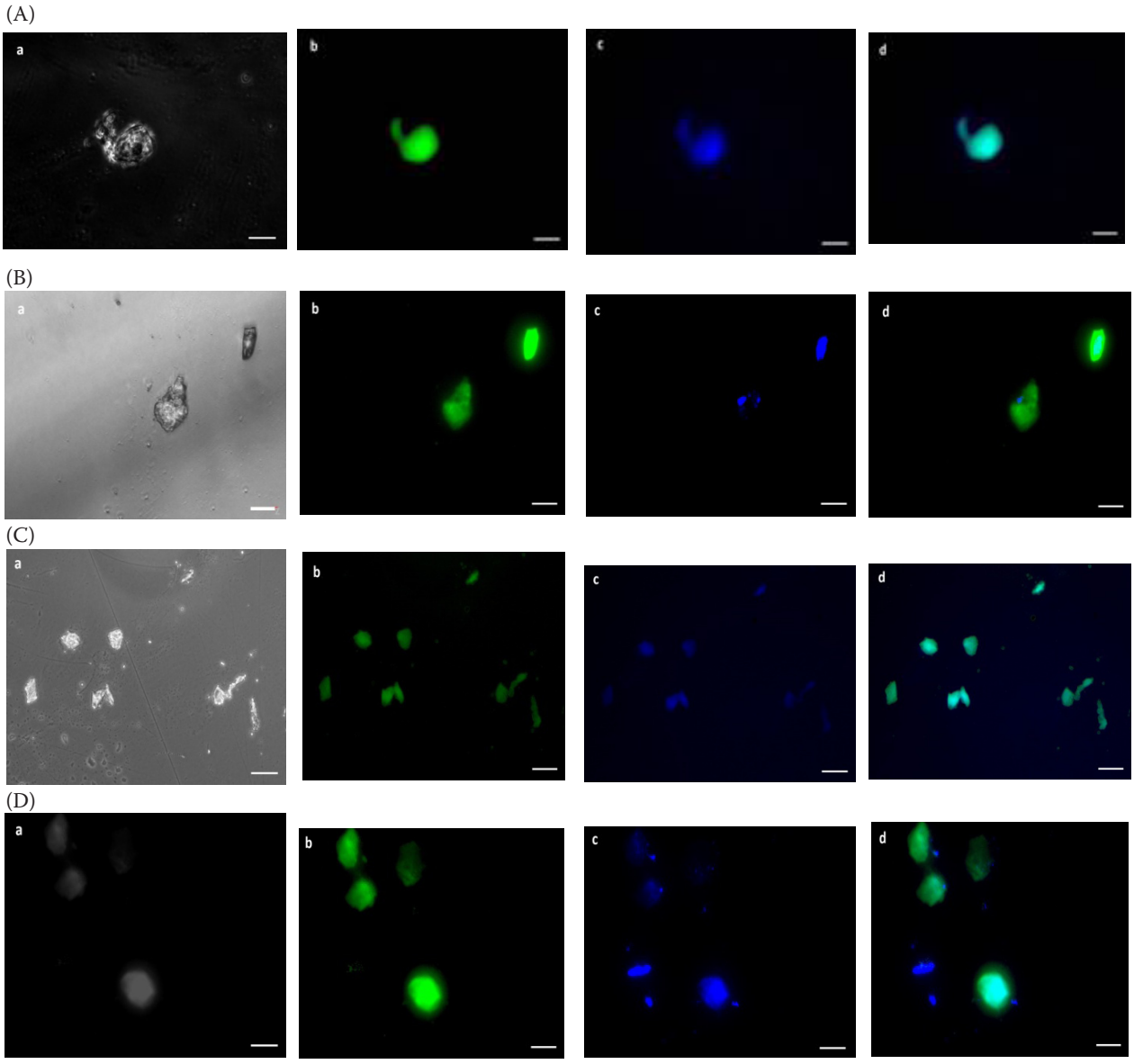

Figure 4. Positive immunofluorescence staining of spermatogonial stem cells for (A) *OCT4*, (B) *SSEA1*, (C) *ITGB1* and (D) *PGP9.5* on day 65 of culture: (a) under bright field; (b) expression of specific marker proteins; (c) location of the nuclei stained by DAPI; (d) merged images of b and c (scale bar: 50 µm)

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of Han et al. 2009, who reported that pig SSC colonies grew bigger with time and their average diameter reached 110 ± 4.5 µm by 6–7 days of culture. Lim et al. (2014) also opined that mouse SSCs proliferated continuously and maintained similar size and morphology till 60 days of culture. However, Pramod and Mitrta (2014) observed that the size of the goat SSC colonies increased till 10–12 days and could be maintained till one and a half months. The number of colonies was found to decrease significantly ($P < 0.01$) from 99.17 ± 4.10 on day 9 to 30.50 ± 1.13 on day 65 of culture and corroborates well the findings of Kala et al. (2012), who reported that the number of colonies declined in 4 weeks of culture when they cultured buffalo PSSCs in different culture media. This might be due to the inability of *in vitro* culture conditions to provide a proper microenvironment to maintain spermatogonial stem cells for longer duration of time.

Alkaline phosphatase staining

Enriched PSSC colonies from all the days of culture were found to be positive for alkaline phosphatase staining (Figure 3), which agrees well with the previous research findings showing that PSSCs strongly expressed alkaline phosphatase (Han et al. 2009; Shi et al. 2015).

Immunofluorescence staining

In the present study, *OCT4*, *SSEA1*, *ITGB1* and *PGP9.5* were taken for immunofluorescence analysis and positive staining for all these pluripotent markers was observed in PSSC colonies derived from days 9, 30 and 65 of culture (Figure 4). *OCT4* is a transcriptional factor for regulating self-renewal and pluripotency and is highly expressed in pig embryonic stem cells (Brevini et al. 2007). It is also expressed in undifferentiated spermatogonia (Luo et al. 2006). *SSEA1* is a biochemical marker for embryonic stem cells in mice and is also expressed in porcine spermatogonial stem cells (Takagi et al. 1997). *PGP9.5* and *ITGB1* are specific for porcine SSCs (Kon et al. 1999). A positive immunofluorescence staining of Sertoli cells with Vimentin was observed in the present study (Figure 5). Scarpino et al. (1998) also stated that Vimentin is a cytoskeletal protein and a marker for Sertoli cells. The results of immunofluorescence analysis in the present study showed that PSSCs were positive for *OCT4, ITGB1, SSEA1* and *PGP9.5* and negative for Vimentin, which served as a negative control, and which was supported by the findings of earlier works in pig SSCs (Han et al. 2009; Shi et al. 2015). Similarly, the Sertoli cell feeder layer was found to be negative for all the antibodies under consideration except Vimentin.

As a negative control, staining was performed without primary antibody, showing no specific staining by the secondary antibody (Figure 6). Though the shape of the cells was not found to be maintained on day 65 of culture, it remained positive for alkaline phosphatase and immunofluorescence staining indicating no loss of pluripotency. Shi et al. (2015) opined that the shape of the porcine SSCs was maintained after 45 days, however they lost

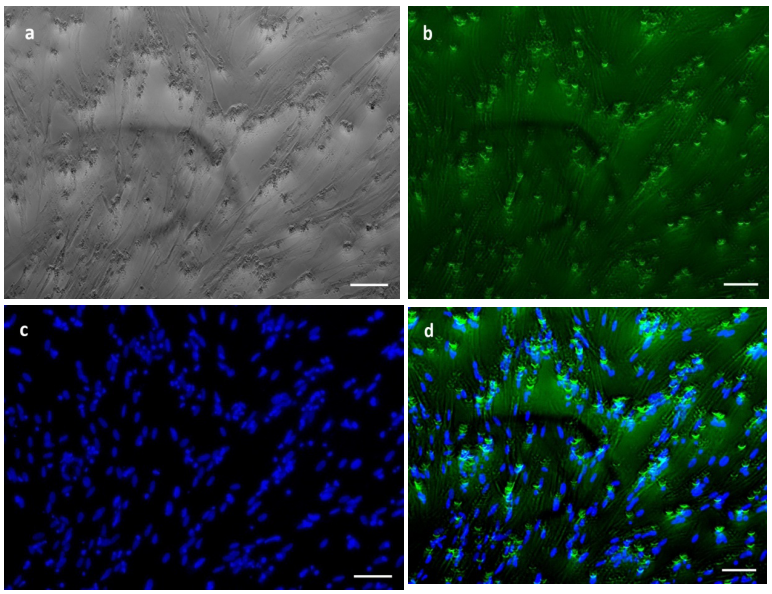

Figure 5. Immunofluorescence staining of Vimentin by Sertoli cells: (a) under bright field; (b) expression of specific marker proteins; (c) location of the nuclei stained by DAPI; (d) merged images of b and c (scale bar: 100 µm)

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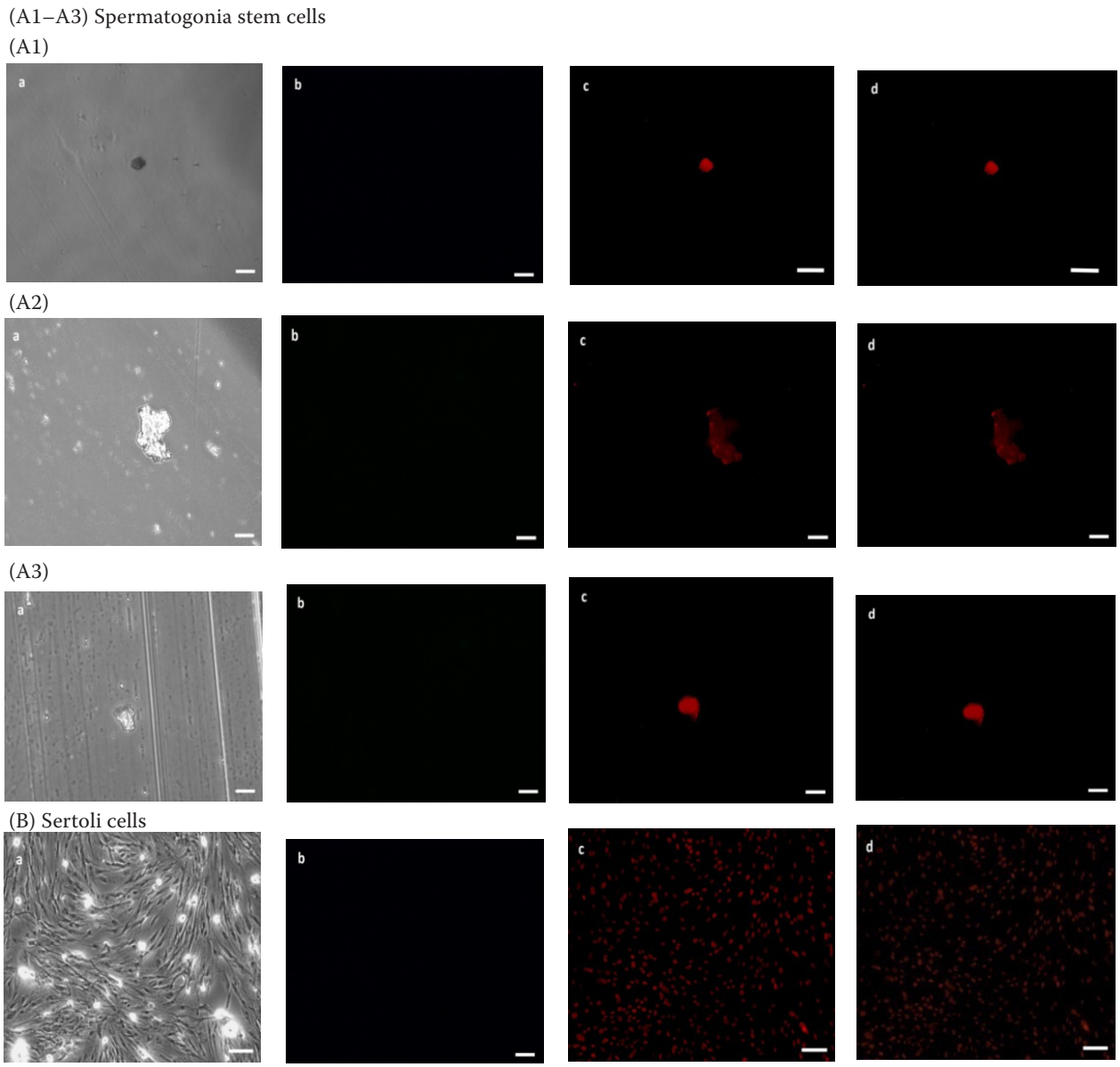

Figure 6. Secondary antibody control for (A1–A3) spermatogonial stem cells: (A1) FITC Sheep anti-Rabbit for *OCT4* and *PGP9.5*; (A2) Alexa Fluor 488 Goat anti-Mouse for *SSEA1*; (A3) Alexa Fluor 488 Goat anti-Rabbit for *ITGB1;* and (B) Sertoli cells (Alexa Fluor 488 Goat anti-Mouse for Vimentin): as a negative control, staining was performed without primary antibody, showing no specific staining by the secondary antibody – (a) under bright field; (b) no expression of specific marker proteins; (c) location of the nuclei stained by propidium iodide; (d) merged imagesof b and c (scale bar: 100 µm)

their pluripotency. In this study we could clearly demonstrate that the present culture media can help maintain the SSCs at least up to two months when co-cultured with Sertoli cells.

SYBR Green based real-time PCR for quantification

The comparative expression of marker genes *OCT4*, *SOX2*, *c-Kit* and *PPARγ* on days 9, 30 and 65 of culture was studied. The relatively quantified (RQ) values obtained on day 9 of culture were used for calibration and were considered as 1.00 to compare the expression on days 30 and 65 of culture. The RQ values of *SOX2* and *OCT4* were 0.54 and 0.96 on day 30 culture, respectively, and 0.00 for both on day 65 of culture. Hence, the expression of *SOX2* and *OCT4* was found to be higher on day 9 than on day 30 of culture. However, the expression was reduced considerably on day 65 of culture

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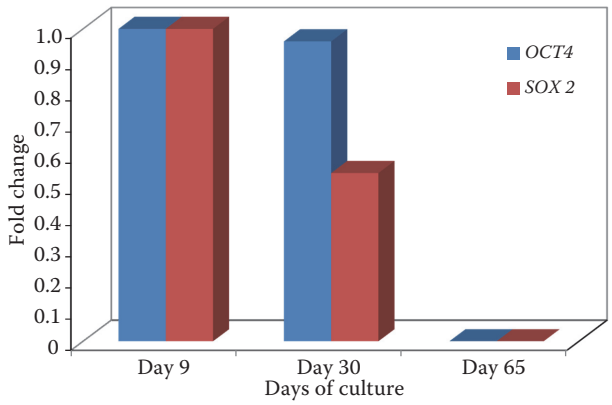

Figure 7. Expression level of spermatogonial stem cells for specific pluripotent marker genes on different day of culture

(Figure 7). The qPCR analysis showed that the cultured PSSCs expressed only *OCT4* and *SOX2*, but not *c-Kit* and *PPARγ*, which are markers of differentiating spermatogonia. The specific products were also obtained for each gene on electrophoresis (Figure 8). Zhang et al. 2011 reported that undifferentiated and differentiated spermatogonia differ in the expression of *c-Kit* and are expressed by differentiated spermatogonia. *c-Kit* and *PPARγ* have been markers for lost SSC pluripotency and *PPARγ* was expressed in differentiating SSCs into adipocytes in pig as reported by Shi et al. (2015). The expression of *OCT4* and *SOX2* by PSSCs as observed in the present study strongly indicates that they are undifferentiated cells.

CONCLUSION

The results of the study successfully depicted the *in vitro* culture conditions for long-term maintenance of SSCs of Doom pigs without differentiation. The SSC population was enriched to eliminate the effect of other different testicular cells and also differentiated germ cells. Finally, the cell population with a high concentration of SSC was identified while we tried to develop culture conditions to maintain their stemness for longer duration of time. In the present study, it was observed that the enriched SSCs could be maintained up to two months. The isolation, enrichment and long-term culture method for SSCs from Doom pigs will provide opportunities for better reproductive management as well as conservation of these pigs.

Conflict of interest. The authors declare no conflict of interest.

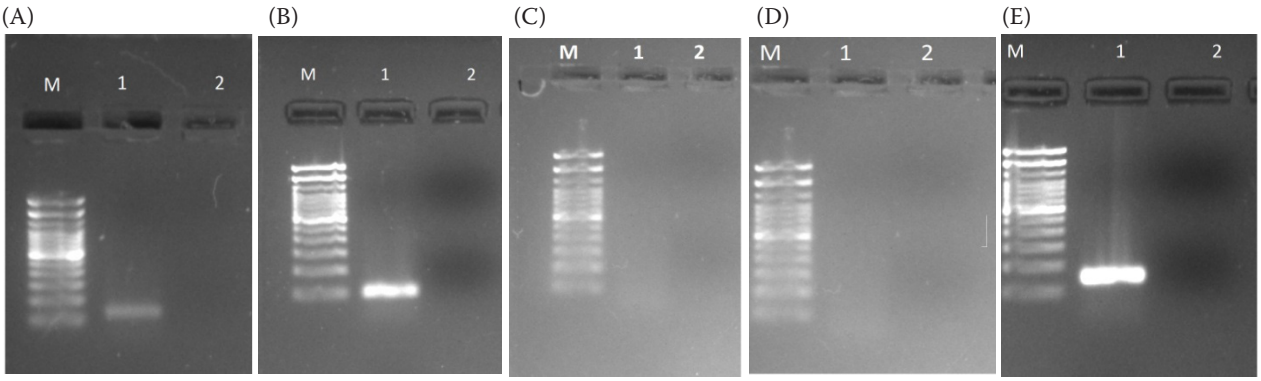

Figure 8. Real-time PCR results for various spermatogonial stem cells markers: (A) *SOX2*; (B) *OCT4*; (C) *c-Kit*; (D) *PPARγ* on electrophoresis; (E) *ACTB* (house keeping gene)

M = 100 bp DNA marker; line 1 = spermatogonial stem cell; line 2 = non-template control

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