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In-Vitro Culture and Characterization of Spermatogonial Stem Cells (SSCs) in Pre-Pubertal Mice

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A B S T R A C T

Spermatogonial Stem Cells (SSCs) are unipotent adult stem cells, defined as the undifferentiated stem cells located along the basement membrane of the seminiferous tubules. This present study is aimed to carry out the culture and characterization of SSCs in pre-pubertal age group of mice. Isolation of SSCs was done by two-step enzymatic digestion method and was seeded in a six-well plate. In primary culture (P0), colony forming unit was observed on day four post incubation. Mice Spermatogonial Stem Cells (mSSCs) adherence was observed on day eight post incubation. On twelve day post incubation, the cells were subcultured for further expansion. In the subsequent passages (P1 and P2) though a heterogenous morphology was observed, most of the cells showed spindle shaped structure. The pre-pubertal mSSCs were positive for alkaline phosphatase activity at P1 and P2 showed positive expression for the transcription factor, Oct4 was indicated by intranuclear green fluorescence. Flowcytometric analysis revealed that the mSSCs in the pre-pubertal age groups showed expression for the SSEA1 marker.

Introduction

Stem cells in the male germ line were called spermatogonial stem cells (SSCs) and considered to be single cells derived from gonocytes at the start of spermatogenesis, which occurs postnatally during the pre-pubertal period (Wrobel *et al.*, 1995; Oatley *et al.*, 2002). SSCs were unipotent adult stem cells, defined as the undifferentiated stem cells located along the basement membrane of the seminiferous tubules (Senger, 2005). A continuous supply of differentiating germ cells is essential for spermatogenesis. Therefore, SSCs must have the capacity for self-renewal and maintenance of the undifferentiated state. SSCs had two fates, cell division could give rise to either more undifferentiated SSCs (self-renewal) or to a differentiated cell population committed to enter meiosis and became mature spermatozoa (de Rooij and Russell, 2000). SSCs could, therefore, serve as a useful resource for the preservation of germ plasm of

threatened and endangered mammals (Pukazhenthi *et al.,* 2006). The present study is aimed for *in-vitro* culture and characterization of SSCs in pre-pubertal mice.

Materials and Methods

Testis samples were collected from eight pre-pubertal (2–4 weeks) mice. The mice were purchased from the Laboratory Animal Medicine unit, Madhavaram Milk Colony, Tamil Nadu Veterinary and Animal Sciences University, Chennai-600 051.

The decapsulated testes were minced using BP blade on sterile Petridish in Dulbecco's Modified Eagle's Medium with antibiotic solution. The minced testes were placed in 5 ml of DMEM containing 0.5 mg/ml collagenase type I and the seminiferous cords were incubated in 20 ml of DMEM containing 0.5 mg/ml trypsin and 1 µg/ml DNase (Bellve *et al.,* 1977). The dispersed seminiferous cells were then washed twice, resuspended in DMEM containing 5 per cent Fetal Bovine Serum (FBS), filtered through cell strainer (40µ), and the cell concentration was determined with a hemocytometer.

Isolated SSCs were seeded at a density of 7 × 10^4 cells per well in a six-well plate in (DMEM-HG) (45 ml DMEM+5 ml of 10per cent FBS+0.5 ml antibiotics+0.5ml NEAA) (Kanatsu-Shinohara *et al.,* 2008). The six-well plates were incubated at 37°C with 5 per cent CO_2 level. On day 0, four hours after seeding in six-well plate, the floating cells were aspirated and transferred to a second plate and then cultured overnight until the remaining somatic cells fully attached to the bottom of the plate and began to grow. On day one, the floating cells were then collected by gentle pipetting and transferred to a third plate in DMEM, and then incubated. This method is called differential adherence technique and is done for enriching the stem cell population.

After 60–70 per cent confluency, the cells were detached from the six-well plate by using 0.25 per cent trypsin/EDTA for 1-2 minutes at 37°C and seeded into a new plate at 1:2 for subsequent passages. The cells were cultured up to P2 level in this study.

Alkaline phosphatase

For assessing alkaline phosphatase activity in putative SSC, the cells were fixed with 4 per cent paraformaldehyde and stained immunocytochemically using the AP staining kit as per the manufacturer's protocol. The AP activity was estimated by visual analysis of the stained cells (Ju *et al.,* 2008).

Flow cytometry

Characterization of mouse SSCs by positive (SSEA1+) and negative (CD34-) surface markers was performed by using FACS™ using mouse SSEA1 primary antibody (1 in 500 dilution) as per Kannan *et al.,* (2016). Immunostaining was carried out for CD34 surface marker by the same procedure. Flow cytometric analysis was performed on Becton, Dickinson FACS using a 488nm-argon-ion laser and 632nm red LASER for excitation. In the histogram, P1 represented the unstained population and P2 was the stained population *i.e.* positive percentage of cells for the specific marker.

Immunostaining

Cultured cells in 12 well plates were washed in Dulbecco's phosphate buffered saline (DPBS) twice and the cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature. After blocking, 500 µl of Oct4 primary antibody was added to the well and stored at 4°C over night. The cells were washed twice with DPBS and then the secondary antibody (i.e.) anti rabbit-FITC was added to the well and incubated for 90 minutes. Secondary antibody was removed

and the cells were again washed with DPBS twice. To this, 400µl of 4'-6- diamidino-2-phenyl indole (DAPI) (1 in 1000 dilution with PBS) was added and kept at room temperature for 10 minutes. DAPI was removed and PBS washing was done 2 times. To this, 1000 µl of PBS was added and kept at 4°C until the plate was examined under fluorescence microscope (Violet Beulah *et al*., 2016).

Results and Discussion

Culture and differential adherence selection

Viable mSSCs were seeded at the density of 7×10^4 cells per well in a six-well plate for the primary culture (P0) in DMEM and incubated at 37^0C with 5 per cent CO_2 in the present study as per the reference of Tiptanavattana *et al.,* (2013) in Feline.

On day 0, four hours after seeding in six-well plate, the floating cells were aspirated and transferred to a second plate and then cultured overnight until the remaining somatic cells got fully attached to the bottom of the plate and began to grow.

On day one, the floating cells were then collected by gentle pipetting and transferred to a third plate in DMEM, and then incubated. A similar procedure has been adapted by Ogawa *et al.,* (2004), Shi *et al.,* (2006) in mice and Liu *et al.,* (2011) in human.

The fibroblasts and other cells started adhering within four hours after seeding. This procedure helped in enriching the SSCs by collecting the floating germ cells and transferring them to new plates subsequently. This helped in eliminating the somatic cells.

On day four, mSSCs were observed in different forms such as single, paired and small clusters (colony forming unit) of three to five cells were observed (Figure 1).

The colonies were mostly single, oval and biconvex. Some of the colonies were observed paired, catenarian and cluster forms. On subsequent days of culture, the number of mSSCs in colony increased and therefore the size of the colonies also increased and attained a three-dimensional (3D) shape on day seven post incubation.

Subculture and expansion

The cultured cells from P0, on day twelve post incubation with 50 to 60 per cent confluency were trypsinized and seeded into a new plate at 1:2 ratio for passage P1. Most of the seeded cells were adherent on the first day itself. Though the cells exhibited different morphology (viz., round, stellate, polyhedral, and triangular) the stellate shaped cells were found to be more. A similar observation has been made by Jeong *et al.,* (2003) in mice and Wang *et al.,* (2015) in pig SSCs.

On day four post incubation, the cells attained about 60 to 70 per cent confluency at passage 1 (P1) level (Figure 3). The cell yield at P1 level was found to be 4.5×10^6cells which is in accordance with the findings of Kanatsu-Shinohara *et al.,* (2003) in mouse.

The cells were subcultured to passage 2 (P2) level and about 70 to 80 per cent confluency was observed in day four post incubation. The cell yield was found to be 4.9×10^6cells (Figure 2). This is in accordance with the findings of Kossack *et al.,* (2009).

Characterization of MSSCS

Alkaline phosphatase staining

In pre-pubertal age groups, strong Alkaline phosphatase activity was observed in mSSCs at passage 1 (P1) when compared to passage 0 (P0). This is in agreement with Van Der Wee *et al.,* (2001) and Stefkova *et al.,* (2015) in mouse.

Fig.1 photomicrograph of pre-pubertal mSSCs at P0/Day4 showing colony forming units (arrows) x 200 Pr- paired cells S- single cell

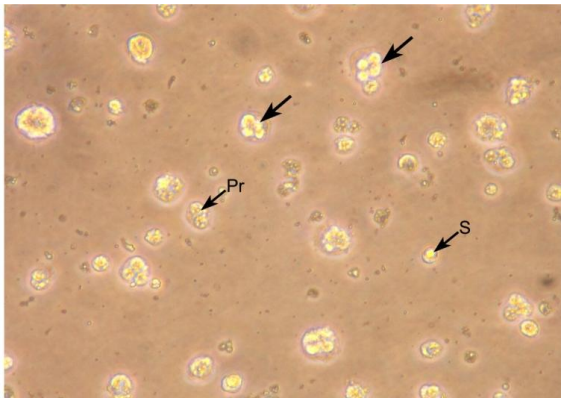


Fig.2 Photomicrograph of pre-pubertal mSSCs at P2/Day4 showing 70-80% confluency X200

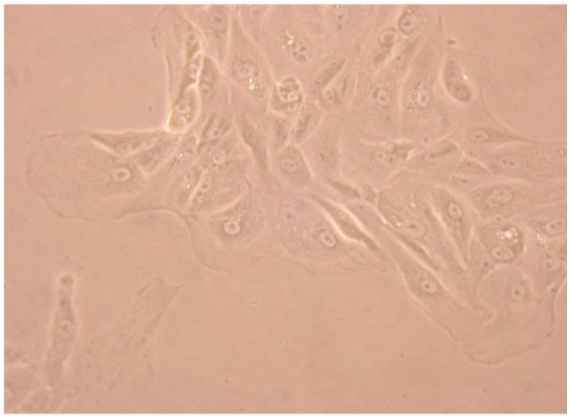


Fig.3 Photomicrograph of pre-pubertal mSSCs at P0 showing positive expression for Oct4 X 200

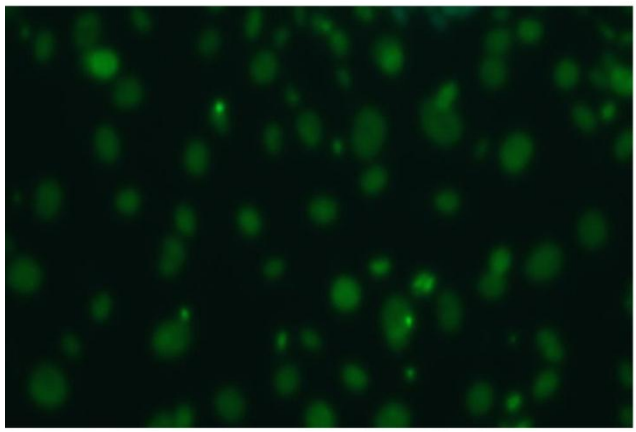
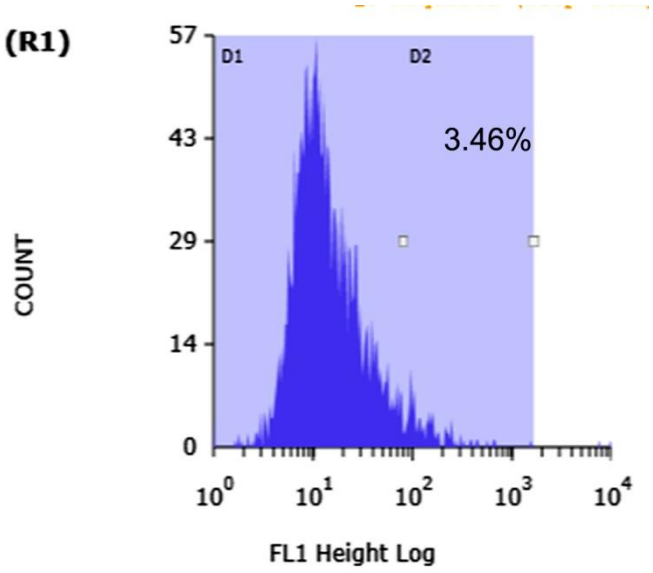


Fig.4 Histogram representing the flowcytometric analysis of pre-pubertal mSSCs at passage 1 (P1) level using SSEA1 marker



Expression of transcription factors

In the present study, the mSSCs from pre-pubertal age groups at passage 0 (P0) were positive for the transcription factor Oct4 (Figure 6) which was confirmed by intranuclear green fluorescence. Oct4 which

indicated that the mSSC colonies were in undifferentiated stage and had pluripotent characteristic as reported by Matsui *et al.*, (1992) and Resnick *et al.*, (1992) in mouse and Panahi *et al.*, (2011) in bovine and is required to govern the pluripotency of cells (Reed and Jhonson, 2008).

Flow cytometry

SSEA1 marker

The cultured mSSCs in pre-pubertal age groups exhibited positive expression for SSEA1 by flowcytometry. In pre-pubertal age group, the primary culture (P0) showed 2.37 ± 0.302 per cent positive for SSEA1 and in passage 1 (P1) 3.46 ± 0.139 per cent positive for SSEA1 (Figure 4). In the present study, an increasing trend was observed between passage 0 (P0) and passage 1 (P1) in pre-pubertal mice. A similar observation has been made by Lim *et al.*, (2012) in humans who demonstrated that in the flow cytometric analysis, the number of SSEA-4 positive cells in SSC clumps is greater at passage 5 (8.45 per cent) than at passage 1 (2.64 per cent). The increase in SSEA1 level indicated that the pluripotent nature is maintained up to passage 1 (P1) in the present study.

CD34 Marker

In pre-pubertal age groups, passage 0 and passage 1 showed little expression for CD34 by flowcytometry. This is in accordance with the findings of Kubota *et al.*, (2003) who stated that the SSCs showed very low or no expression of CD34 markers.

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