Original Article

Spermatogonial Stem Cells Protein Identification in In Vitro Culture from Non-Obstructive Azoospermia Patient

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Abstract

***Background*: Spermatogonial stem cells (SSCs) are classified as a unique adult stem cells that have capability to propagate, differentiate, and transmit genetic information to the next generation. Studies on human SSCs may help resolve male infertility problems, especially in azoospermia patients. Therefore, this study aims to propagate SSCs *in-vitro* with a presence of growth factor and detect SSC-specific protein cell surface markers.**

***Methods:* The sample was derived from non-obstructive azoospermic (NOA) patient. The disassociation of SSCs was done using trypsin. Specific cultures in serum-free media with added basic fibroblast growth factor (bFGF) were developed to support self-renewal division. This undifferentiated protocol was performed for 49 days. Cells were analysed on days 1, 7, 14, 21, and 49.**

***Results:* Human SSCs began to aggregate and form colonies after 14 to 21 days in specific culture. Then, the cells were successful expanded and remained stable for a duration of 49 days. Four specifics markers were identified using immunofluorescence in SSCs on day 49: ITGα6, ITGβ1, CD9, and GFRα1.**

***Conclusion:* This approach of using *in vitro* culture with additional growth factor is able to propagate SSCs from non-obstructive azoospermia patient via detection of protein cell surface markers.**

Keywords: *spermatogonial stem cells, cell protein marker, non-obstructive azoospermia, in vitro*

Introduction

The number of male infertility cases such as azoospermia, oligozoospermia, and asthenoteratozoospermia have been increasing every year. Knowledge about the mechanism of spermatogenesis is limited and maintaining the optimal culturing condition with growth factors in SSCs is challenging. SSCs have the ability to undergo self-renewal and differentiated germ cells into spermatozoa throughout life (1). Kevin et al. (2) stated a unique characteristic of SSCs is that they are the only adult cell type that can continuously self-renew and transmit genetic information to future generations. The genetic integrity of SSCs has to be maintained along the culturing system in order to guarantee a correct transmission of genetic information from this generation to the next.

The establishment of an optimal culture medium for SSCs has been eagerly anticipated in recent years. Culture conditions needed for long-term stabilisation of SSCs usually consist of culture environment, serum factor, and growth factor alone. They may also include combinations such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), glial cell line-derived neutrophic factor (GDNF) and leukemia inhibitory factor (LIF) (3, 4, 5, 6). Sato et al. (7) proved that the utility of Knockout Serum Replacement (KSR) as a cell culture supplement for culturing mouse SSCs. Knockout

Dulbecco's Modified Eagle Medium (DMEM) used with KSR significantly reduces embryonic stem cell (ESC) differentiation, growth, and maintenance of undifferentiated ESCs. Kossack et al. (8) discovered that the culture of human testicular cells under ESCs conditions resulted in the formation of SSC colonies. Shinohara et al. (9) reported that when bFGF was added to the culture medium, the SSCs were able to keep in culture for more than five months. Bart et al. (10) demonstrated that development of a serum free and essential growth factor was critical to the establishment of long-term SSCs cultures. Chunhui et al. (11) found that human embryonic stem cell (hESC) media can be maintained in bFGF or combination with other growth factors in a serum replacement media. A combination of growth factors such as GDNF, bFGF and EGF has improved propagation of SSCs in non-obstructive azoospermia (NOA) and obstructive azoospermia (OA) patients in long-term culture (12). The use of growth factors can be improved and allow SSCs to be cultured for longer periods without altering their undifferentiated properties, thus providing an unlimited supply of SSCs.

The molecular characteristics of SSCs in adult primate testes have been identified recently (13, 14). However, there is limited knowledge about the characteristics of human spermatogonia. Until now, expression of Integrin alpha-6 (ITGα6), CD133, GFR-alpha-1 (GFRα1), GPR-125, Integrin beta-1 (ITGβ1) MAGE-4, PLZF, SSEA-4, CD9 and CD90 has been reported in human spermatogonia (15, 16, 17, 18). However, the identity and characteristics of SSCs in adult human testes remain poorly understood.

The current study was specifically conducted to evaluate the culture system of SSCs derived from testis biopsy of NOA patient and to detect specific protein surface markers by immunofluorescence (IF). Culturing using hESC media supplemented with bFGF has indeed enhanced proliferation and the growth of SSCs and was demonstrated with the presence of ITGα6, ITGβ1, CD9 and GFRα1. SSCs are an important target cell for restoring male fertility, particularly for NOA patients. Hence, the detection of SSCs population in NOA patient using *in vitro* culture gives hope for them to have their own offspring.

Materials and Methods

Cells preparation for culturing

Testicular tissue was collected by TESE procedure (testicular sperm extraction) from NOA patient referred to *in vitro* fertilisation (IVF) clinical center, National University Malaysia. The biopsied testes tissues were washed by centrifugation at least twice with commercial IVF sperm washing media (Irvine Scientific, USA). The testicular tissue was minced into small pieces and digested with 2 mL of 0.25% trypsin (Invitrogen, UK) for 10 min in 37°C temperature and 5% CO_2 incubator. 2 mL of hESC media was added to stop the activation of trypsin. Then, the disassociated cells were collected via centrifugation at 800 rpm for 5 min. Supernatant was discarded and the pellets were resuspended in fresh hESC media and cultured in 24-well plate.

Culture technique

The disassociated cells were cultured in hESC media with 80% Knockout DMEM (Gibco, UK), 20% Knockout Serum Replacement, 1% non-essential amino acids, 0.1% gentamicin, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and growth factor of 80 µl bFGF (Gibco, UK). The medium was sterilised by filtering using 225 mL 0.2 µm cellulose acetate filter unit and stored at 4oC until needed for a maximum of three weeks. The SSCs culture media was changed every 2-3 days. The cells were cultured up to 49 days and visually analysed on days 1, 7, 14, 21 and 49 using a Primo Vert inverted microscope (Carl Zeiss, Germany) using ZEN lite 2012 software under 200× magnification to investigate the propagation activity of SSCs. SSCs that have propagated in cell culture for 49 days without differentiating are referred to as the SSCs population.

Immunofluorescence (IF) staining

The cells were cultured up to 49 days and analysed on days 1, 7, 14, 21 and 49 by an Olympus IX81 immunofluorescence (IF) (Olympus, USA) microscope using Metamorph imaging software. The higher-power magnification (400×) was used for IF detection. SSCs that have propagated and detected in IF staining were represented as SSCs population. Cell preparations were preserved by fixation prior to immunofluorescence staining for detection of specific cell surface markers. This was essential to protect cell membranes. The

culture medium was discarded from the well and cells were washed with phosphate buffered saline (PBS). Then, the cells were fixed in culture well with 4% paraformaldehyde (PFA) for one hour at room temperature. The 4% of paraformaldehyde (PFA) was prepared by heating (65°C) the mixture of 4 g of PFA and 100 ml of PBS on heating plate and constantly stirred the mixture until totally diluted. Later PFA was removed and cells were washed three times with PBS supplemented with 5% fetal bovine serum (FBS). The cells were then stained with primary antibody. Anti-ITGα6 at a dilution 1:1000, anti-CD9 at a dilution 1:50, anti-ITGβ1 at dilution 1:500 and anti-GFRα1 at dilution 1:1000 were added to the culture well in a specific dilution of PBS + 5% FBS based on the dilution recommendation given in the data sheet of the product (Abcam, UK). The cells were incubated at room temperature for one hour. The primary antibody was removed and cells were washed three times with PBS + 5% FBS. Secondary antibodies (fluorescent conjugate), goat anti-mouse IgG2b heavy chain, FITC (1:1000) and goat anti-rabbit Texas-red (1:1000) were added. The cells were incubated for one hour at room temperature with the well plate covered with aluminium foil to protect from the light. Then, the secondary antibody was removed and the cells were washed three times with PBS + 5% FBS. The secondary antibody was selected based on isotype of primary antibody and the animal of origin. 4'6'-Diamidino-2 phenylindole, DAPI (1 mg/1 ml) was used for nuclear visualisation. DAPI was added at the end of the process. The cells were then brought to the fluorescence microscope for photo micrograph images to be captured.

Results

Cell adhesion and morphology of SSCs during the culture

After a simple trypsin digesting protocol on testicular tissues and culturing for 24 hours, a little number of SSCs adhered to culture plates. Under an inverted microscope, the SSCs were observed as round or oval shape with large nucleus and little cytoplasm after one week of culture. Based on morphology, human SSCs started to aggregate and form small clusters on day 14 of culture in hESC media with bFGF. The medium was changed every 2–3 days up to 49 days (Figure 1). The morphology appearance of undifferentiated SSC colonies became more typical. The cells were propagated from a single cell suspension and formed colonies, showing that the propagation activity of SSCs was expanded. Later, the efficacy of culture medium was analysed via immunofluorescence (IF) detection on days 1, 7, 14, 21 and 49.

Immunofluorescence (IF) Detection of SSC markers: ITGα6, ITGβ1, CD9 and GFRα1

IF was performed to detect the population of SSCs in the specific culture condition on days 1, 7, 14, 21 and 49. Using indirect method of IF staining, small populations of ITGα6, ITGβ1, CD9 and GFRα1 positive cells were showed only on day 49 of culture. Positive populations of cells appeared on certain location and not over the entire culture well. The IF was observed in cell membrane, which was a typical staining pattern of spermatogonia. IF detection of SSCs after 49 days in culture revealed that the cells were maintained in an undifferentiated state in hESC media.

Figure 2A shows ITGα6 was positively detected with the presence of green fluorescent colour while in Figure 2B showed CD9 was positively detected with the presence of red fluorescent colour. As for Figure 3A, green fluorescent colour showed positive ITGβ1 protein cell marker, whereas in Figure 3B, red fluorescent colour showed positive GFRα1 protein cell marker. DAPI staining shown other somatic cells were identified in both of the dishes (Figures 2C and 3C). No staining was observed in the negative control (human normal sperm) and SSC cultures on days 1, 7, 14 and 21.

Discussion

Spermatogonial stem cells (SSCs) are categorised as unique adult stem cells because they can undergo self-renewal and transmit genetic information to the next generation (19). The biological study of human SSCs is a worthy research to understand the basic concept of stem cell regulation and therapy in males with infertility problems, such as non-obstructive azoospermic (NOA) patients. Based on the literature, important elements needed to upgrade SSC research include knowledge of stem cell markers and a method to maintain the stem cells (undifferentiated cells) continuously in culture. This preliminary study was conducted to examine the propagation activity of SSCs and investigate the specific protein cell surface markers in NOA patient using IF staining.

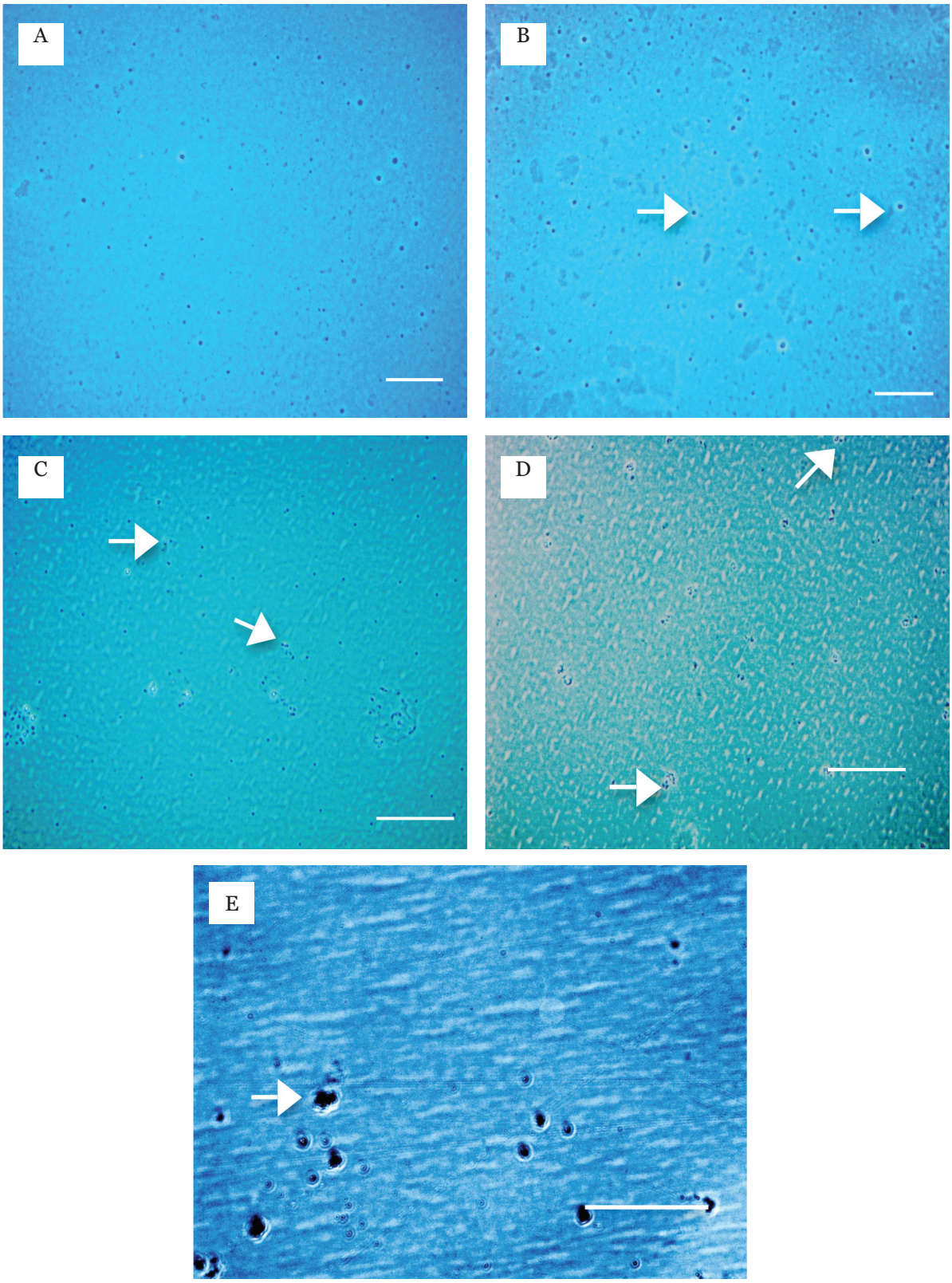

Figure 1: Propagation activity of SSCs *in vitro* culture condition. **A:** 24 h culture, SSCs adhered to the culture plate; **B:** Day 7, SSCs were seen as round or oval shape (arrows); **C:** Day 14, SSC started to form colony (arrows); **D and E:** SSC colonies (arrow) after 21 and 49 days of culture. Scale bars = 324 µm (A), 198 µm (B), 590 µm (C), 408 µm (D) and 324 µm (E). The cells were observed under inverted microscope (200×).

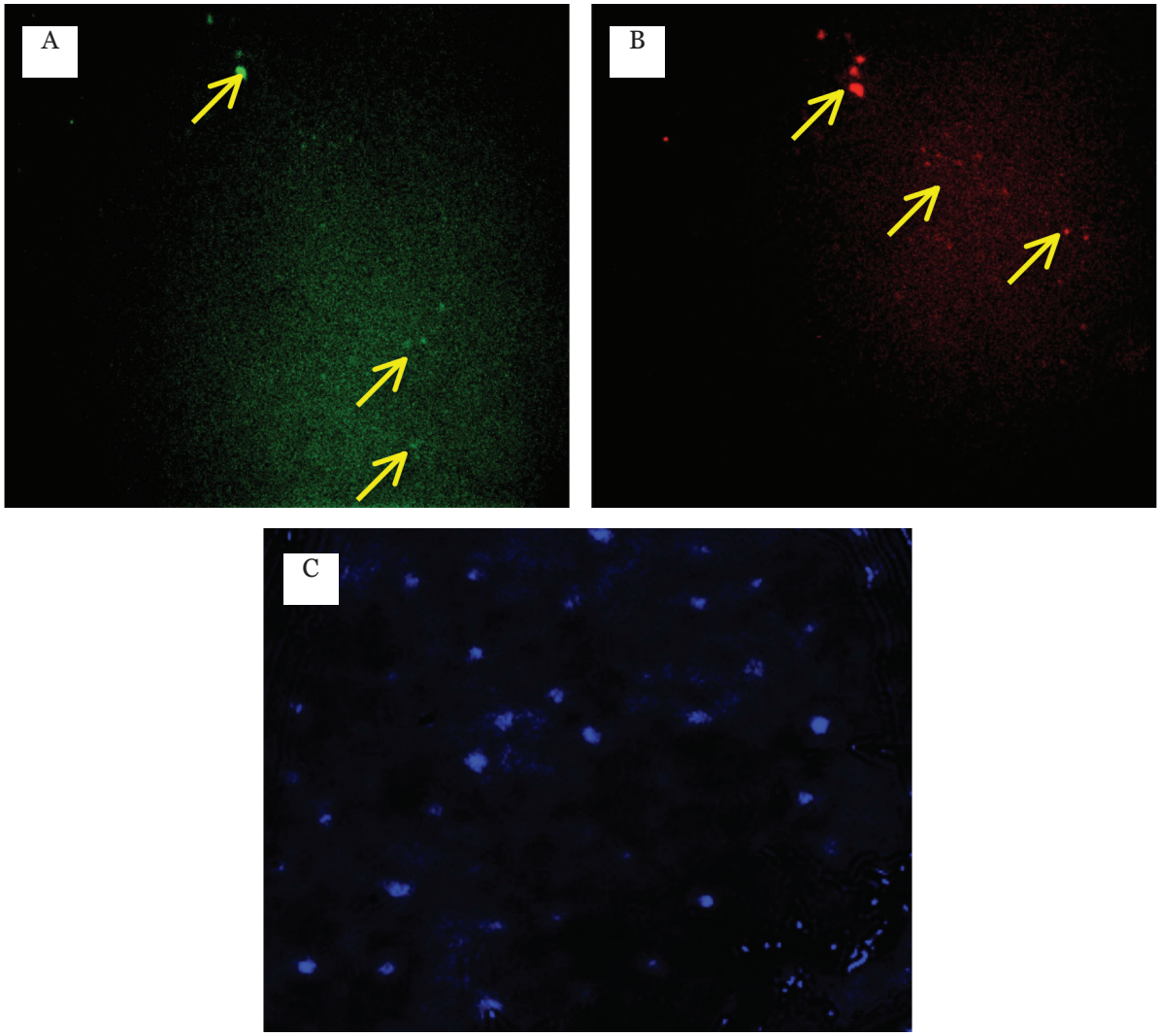

Figure 2: ITGα6 and CD9 positively expressed SSCs population on day 49 of culture (cell surface staining). **A:** Green, stained with ITGα6 with anti-mouse secondary FITC (arrows); **B:** Red, CD9 antibody with anti-rabbit secondary Texas-red (arrows); **C:** Blue, DAPI nuclear stain for other cells. The existence of SSCs were observed under IF microscope (400×). All antibody negative and control were not shown.

Enzymatic digestion process of human testicular tissue is a crucial protocol for preparing single cell suspension, as it may affect cell viability and cause cell injury. In this study, trypsin was used to digest the human testicular tissue. Similar methods were used in other studies using four enzymes to digest human testicular tissue, including collagenase, trypsin, DNase, and hyaluronidase (18). In comparison, the method of digestion in this study was simpler and feasible in operation than others. This type of suspension culture allowed cells to maintain in the culture medium as aggregated and distributed evenly within it. Initially, the aggregated cells appeared first simply as a ball of cells, later increasingly developing into a more complex appearance.

The establishment of specific culture system is important to maintain a specific type of self-renewal division (20). The hESC culture media was used in this study was generally formulated for the development of undifferentiated ESCs, but is also applicable to culture-undifferentiated human SSCs. The Knockout DMEM in hESC media is a special basal medium consists of high glucose designed specifically to improve morphology and maintenance of ESCs as well as

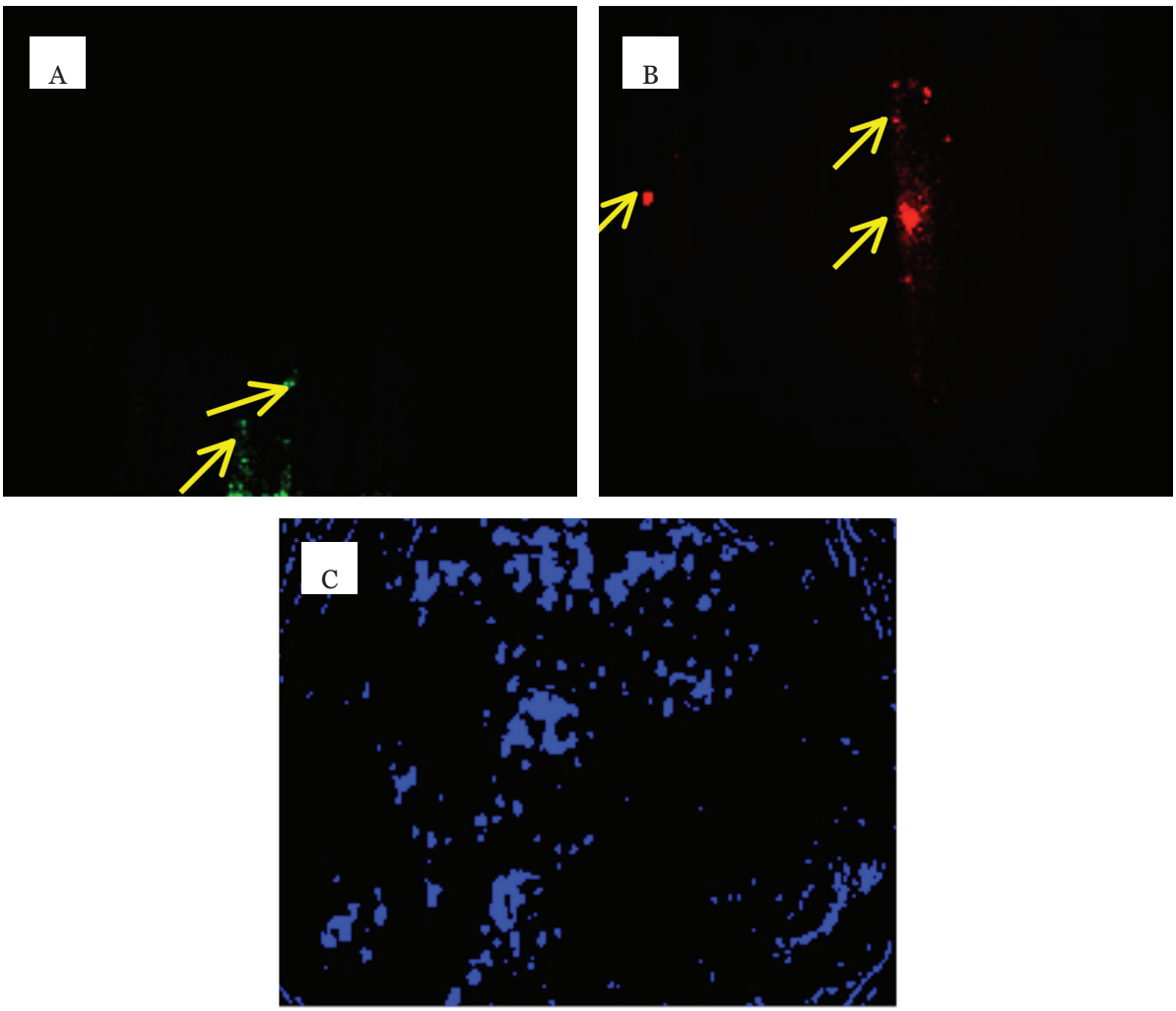

Figure 3: ITGβ1 and GFRα1 positive cell surface protein expressed under inverted microscope on day 49. **A:** ITGβ1 positive cells were detected using IF staining with goat anti-mouse IgG2b green fluorescein isothiocynate (FITC) (arrows); **B:** GFRα1 positively detected SSCs with goat anti-rabbit Texas Red labeled anti- GFRα1 (arrows); **C:** DAPI staining shown positive for other cells. The existence of SSCs were observed under IF microscope (400×). No staining was observed in the negative control using human normal sperm and SSCs culture on day 1, 7, 14 and 21.

SSCs. Serum-free KSR was added as a supplement to the culture condition, providing hormones to trigger cell replication (21). Both reagents were used together to allow for less cell differentiation. The propagation activity of human "SSCs like cells" in this study clearly showed that a single cell suspension developed on day 1, then formed colonies on day 21 and stable colonies on day 49 (Figure 1D). The result of this study is parallel with previous study reported that the long-term culture of SSCs was supported by KSR (21). Sato et al. (7) supported this idea by demonstrated that KSR is vital for testicular organ culture. As shown in this study, the first objective was fulfilled, as the propagation activity of "SSCs like cells" expanded and supported with specific culture system.

Furthermore, the hypothesis of this study was to determine the combination of growth factor and hESC media can support the propagation activity of SSCs population. Based on SSCs morphology from days 1 to 49, the propagation activity of cells gradually expanded from a single cell suspension attached to the plate until aggregation and the formation of stable colonies. It showed that the propagation of "SSCs like cells" was positively observed. Thus, the result of this study suggest

that bFGF alone in culture medium supported the propagation of the undifferentiated SSCs. According to previous studies, the growth factors for example bFGF, LIF, EGF and GDNF were usually added into culture system for a long-term stabilisation of SSCs (20). Therefore, the culture condition with combination of growth factor provides the best condition for SSCs maintenance and propagation for the long term. Liu et al. (19) also supported this study finding, as they reported that two categories were needed for long-term stabilisation of SSCs including added growth factors such as bFGF, GFRα-1, and GDNF; and provided a feeder layers of cells.

In this study, *in vitro* culture of SSCs derived from NOA patient were propagated and maintained their undifferentiated characteristics up to 49 days. The viability of cultured "SSCs like cells" after 24 hours showed few cells attached to the plate. The main factor attributed to this result was the change in environment setting (from *in vivo* to *in vitro*) coupled by the impact of enzymatic digestion. However, on days 7, 14, 21 and 49 of culturing, the propagation activity of "SSCs like cells" was positively seen as they aggregated and formed colonies (Figure 1). This finding of "SSCs like cells" propagation shows that they are possibly adapted to the culture environment and the serum factor necessary for cell growth. The colonisation of human "SSCs like cells" perhaps at a level similar to its natural environment indicates that the culture media served a favourable environment for them. This result, as supported by the previous study, has revealed that the viability of SSCs declined in 24 hours of culture and gradually increased after 48 hours and peak at 72 hours of culture (19).

The undifferentiated protocol was carried out for 49 days and cells were analysed on days 1, 7, 14, 21 and 49 by IF detection for specific protein cell surface markers; ITGα6, ITGβ1, CD9 and GFRα1 to confirm the SSCs population. Indirect immunofluorescence cell technology was used with a green fluorescein isothiocyanate (FITC) and Texas-red as secondary antibodies for labelling. For *in vitro* culture of SSCs on day 49, all four protein surface markers were positively expressed. Under a fluorescent microscope, most of these cells were round and consistent with the shape of SSCs as seen under an inverted microscope (Figures 2–3). This suggests that ITGα6, ITGβ1, CD9, and GFRα1 are useful markers for SSCs as it has confirmed the population of SSCs. This result of localisation and expression of all protein surface markers of SSCs clearly demonstrate the phenotypic and molecular characteristics of human SSCs. This result is supported by other studies which indicated that ITGα6, ITGβ1, CD9, and GFRα1 are commonly found on SSCs. These markers may smooth the progress of communications or interactions between stem cells and their cognate niches which are naturally sited on tissue basement membranes (16, 18, 22, 23, 24). Sadri et al. (25) stated that ITGα6 and ITGβ1 are predominantly expressed by spermatogonia as they act as a specific marker for undifferentiated spermatogonia. Our findings for GFRα1 in SSCs are also supported by He et al. (18), who demonstrated that GFRα1 as expressed in human testes in the plasma membrane of spermatogonia is a marker for mouse SSCs and progenitor cells.

Interestingly, the results show that human SSCs were expressed after day 49 of culture but not detected on days 1, 7, 14 or 21. This result reported that SSCs derived from NOA patient did not merely survive after culturing into specific culture media, but some of them were managed to propagate. One of the major reasons why SSCs were not detected within 21 days of culturing is because of the growth factor alone (bFGF) with some supplements is not enough to trigger the propagation of SSCs for the short-term culture. This may occur due to lack of growth factor contained in whole serum that may cause cells undergo apoptosis in a short-term culture. Initially, the amount of SSCs derived from NOA patient was very low and some of them may have undergone apoptosis, resulting in very slow rate of propagation, as there was no cell-cell interaction to trigger cell propagation. Thus, this condition causes an absence of surface markers detection of SSCs on days 1, 7, 14 and 21.

Furthermore, validated protein markers and biopsy from normal sperm as negative controls also denoted negatively expressed of SSCs population. In normal sperm samples there were no SSCs as the samples were collected through ejaculation. DAPI staining was used to visualise the nucleus of the cells in culture system. DAPI was expressed in all nucleated cells, including somatic cells, and does not specifically represent the SSC population, as the *in vitro* culturing of SSCs derived from NOA patient may contain other types of cells (Figures 2C and 3C).

This technical issue should be taken into consideration when clinically applying human SSCs, as harvesting a large number of testis cells is not expected to be a common practice. This study suggests that hESC media with

combination of bFGF can support a long-term culture of SSCs. ITGα6, ITGβ1, CD9 and GFRα1 are the protein surface markers for human SSCs. Further research should be performed to gain a better understanding of biology and survival requirements for NOA patients.

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Conflict of Interest

None.

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None.

Authors' Contributions

Conception and design: AYAW
Analysis and interpretation of data: AYAW
Drafting of the article: AYAW
Critical revision of the article for important intellectual content: AYAW
Final approval of the article: AYAW, MLMI, RR
Provision of study materials or patients: AYAW, RR
Statistical expertise: AYAW, MLMI
Obtaining of funding: AYAW, MLMI
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