

Identification of Spermatogonial Stem Cell-Like Cell Differentiation *In Vitro* from Azoospermia Patient Using Modified Human Embryonic Stem Cell (HESC) Media

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Abstract

Background: Spermatogenesis is a process where proliferation and differentiation of spermatogonial stem cell (SSC) into haploid sperm cells in the testis. Azoospermia patients have impaired spermatogenesis where they have difficulty to get normal sperm. The purpose of this study is to proliferate and differentiate SSC-like cell in *in vitro* culture from azoospermia patient samples and to determine the pattern of gene expressions involved.

Methods and Results: Testicular biopsy tissue obtained from obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) patients were dissociated and cultured in different human embryonic stem cell (HESC) media added with specific growth factors and reproductive hormones according to the day of culture using 24 well plate dishes. SSC-like cell colonies were formed after 5 weeks of culture. Gene expression analyses were done on day 49 and day 90 using quantitative reverse transcription polymerase chain reaction (q-RT PCR). OCT4 was detected in NOA patient and ITGB1 was identified in OA patient for 49 days of culture. Gene expression for all stages of spermatogenic cells in day 90 were mostly detected in OA patient; ITGB1, SCP3, H2B and TNP1. However, gene TNP1 was only expressed in NOA patient shown spermatid formation stage.

Conclusions: Our result shown that there is a potential to develop sperm *in vitro* culture from testis biopsy of azoospermic patients for further clinical application.

Keywords: Spermatogenesis *in vitro*; Azoospermia; Spermatogonial stem cell; Gene expression

Introduction

Azoospermia is defined as absence of sperm in the ejaculated semen, which results in male infertility. It is present in 1% of all men and 10% to 15% of infertile men [1]. There are two types of azoospermia; Obstructive Azoospermia (OA) and Non-Obstructive Azoospermia (NOA). Intracytoplasmic Sperm Injection (ICSI) has become the breakthrough of male infertility treatment especially for OA cases where sperm derived from the epididymis or testis through sperm retrieval procedure [2]. However, in NOA cases, it is challenging and most difficult to treat. In certain NOA cases, ICSI was done using round spermatid and some eggs have been fertilized and had success in implantation but the live birth outcomes are very poor. Even though both azoospermia cases had fertilization and implantation rates, but they differ in the clinical outcomes [3]. Due to the poor results, *in vitro* culture of immature germ cells to mature stage has been approached to improve the outcome. Spermatogenesis *in vitro* has long been attempted mostly in animal studies, but it remains challenging due to restriction of culture systems. Spermatogenesis process has unique micro-environmental niche that requires growth factors, hormones and somatic cells to achieve development into spermatozoa [4]. Human Embryonic Stem Cell (HESC) media were widely used for embryonic stem cell culture and some of these studies had derived the male germ cells from embryonic stem cells [5-8]. This media could possible initiate the SSC-like cell from adult testicular stem cells. The purpose of this study is to proliferate the SSC-like cell and differentiate into the next stage of spermatogenic cell using modified HESC media. The hypothesis of this study is the combination of growth factor and HESC media can support the propagation activity of SSC-like cells population from testis biopsy azoospermia patients. Gene expression analyses were done to determine the spermatogenesis stages achieved.

Materials and Methods

Tissue preparation

Four azoospermia patients (OA and NOA) had undergone Testicular Sperm Extraction (TESE) procedure under local anesthesia to obtain testicular tissues at IIUM Fertility Centre, Kuantan, Pahang, Malaysia. Patients were informed and had signed a consent form to participate in this study and their personal information had been kept anonymity for confidential purpose. Briefly, the testis biopsies were placed on a 35 mm petri dish and added with handling GMOPS media (Vitrolife, Sweden). The biopsies dissociated mechanically using scissors to obtain testicular cells. The cells were aspirated and placed into a 15 ml conical Falcon tube (Becton Dickinson Labware, USA) and added with sperm washing media (Sage, USA). The samples were centrifuged at 1000 rpm for 10 minutes. The visualized pellets then were washed with red blood cells lysis buffer (Life Technologies, USA) and centrifuged at 1000 rpm for 5 minutes to eliminate the red blood cells. The pellets were added with trypsin enzyme media (Life Technologies, USA) and incubated for 5 minutes at 37°C with 5% CO₂ humidity. The samples were then added

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with sperm wash media and centrifuged at 1000 rpm for 5 minutes. The samples were washed again with the same media to eliminate the unwanted digested debris. Then the visualized pellets were prepared for cell culture.

Cell culture

Patient samples have been divided into two groups. First group with two samples (OA and NOA) were using HESC media consisting of knockout DMEM, knockout serum replacement, L-glutamine, 2-mercaptoethanol, gentamicin and Basic Fibroblast Growth Factor (BFGF) and the second group (OA and NOA) were using the same HESC media with additional of Leukemia Inhibitory Factor (LIF). All preparation samples were cultured in a 24-well plate dishes and incubated at 37°C with 5% CO₂ humidity. The culture media in each sample was replaced with new media on every alternate day. After 49 days of cultured, each of the sample dishes had been extended the culture with additional of recombinant Follicle Stimulation Hormone (rFSH) and testosterone hormone added in each media until 90 days as shown in Table 1.

Gene expression

Each sample on 49 and 90 day of cultures had been collected for RNA extraction using trypsin enzyme. All samples were extracted with Nucleospin RNA XS kit (Macherey-Nagel, Germany). I-script cDNA synthesis (Bio-rad, USA) was used to amplify cDNA from 100 ng RNA testicular cell samples. The gene expression analysis had been done using quantitative polymerase chain reaction (qPCR) (Bio-rad CFX96) for day 49 culture; OCT4, ITGB1, GFR125 (Qiagen) and for day 90 culture; ITGB1, SCP3, H2B, TNP1 (Qiagen). The gene markers selected in this study indicated the stage of male germ cells in spermatogenesis process as shown in Figure 1. The cycle protocol of qPCR had been run as follows; 94°C for 1 minute followed by 40 cycles at 94°C, 30 seconds; 60°C, 30 seconds; and 72°C, 30 seconds.

Statistical Analysis

Normalization of gene expression had been done with housekeeping genes of GAPDH and Beta-actin using $\Delta\Delta Cq$ values of q-RT PCR analysis

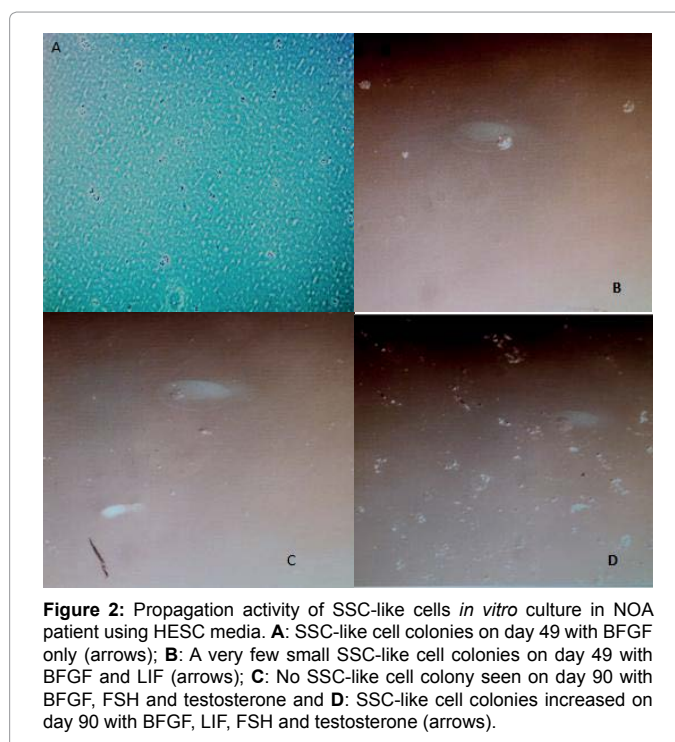
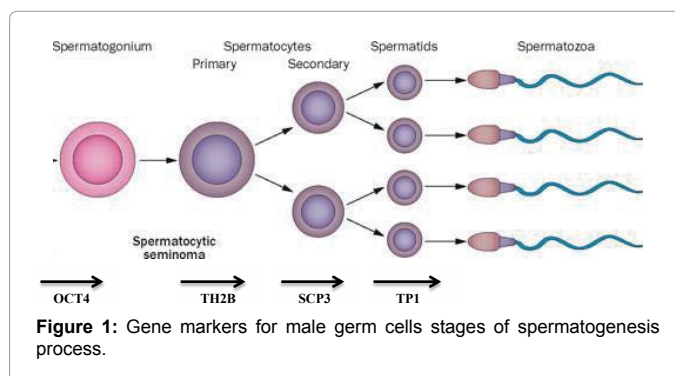
Results

Cell propagation

In NOA patient, SSC was seen as round or oval shape view under inverted microscope after one week of culture using HESC media with BFGF. SSC started to aggregate and form small clusters on day 14 of culture. The SSC colonies were increased after 21 days of culture and stable until up to 49 days. However, there was no SSC colony seen in extended culture of 90 days using the same media added with FSH and testosterone hormones (Figure 2A and 2C). As for NOA sample using HESC media with BFGF and LIF, SSC formed a few small clusters in

Sample	Day 0-49	Day 50-90
NOA	HESC+BFGF	HESC+BFGF+FSH+Testosterone
	HESC+BFGF+LIF	HESC+BFGF+LIF+FSH+Testosterone
OA	HESC+BFGF	IVF+FSH+Testosterone
	HESC+BFGF+LIF	HESC+BFGF+LIF+FSH+Testosterone

Table 1: Media culture preparation according to the day of culture.



after 49 days of culture (Figure 2B). The SSC colonies increase using same media with additional of FSH and testosterone hormones in extended culture until 90 days (Figure 2D).

In OA patient, SSC-like cells formed more colonies on day 49 culture compared to NOA patient. SSC-like cell colonies were more visualized in HESC media with BFGF and LIF than SSC-like cell colonies in HESC media with BFGF only (Figure 3A and 3B). After 49 days of culture, the HESC media with BFGF had been changed into IVF media and had been added with FSH and testosterone hormones while the HESC media with BFGF and LIF were maintain with additional of FSH and testosterone hormones until 90 days of culture. Our result shown SSC-like cell colonies were increased in both of the extended culture (Figure 3C and 3D).

Gene expression analysis

In NOA patient cultured for 49 days, only gene of OCT4 was expressed in HESC media with BFGF only (Graph 1). No gene was expressed in HESC media with BFGF and LIF. After 90 days of extended

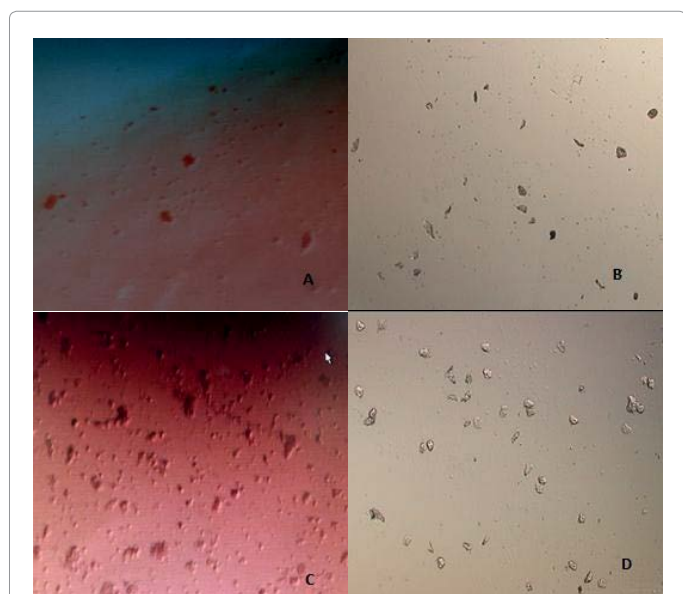
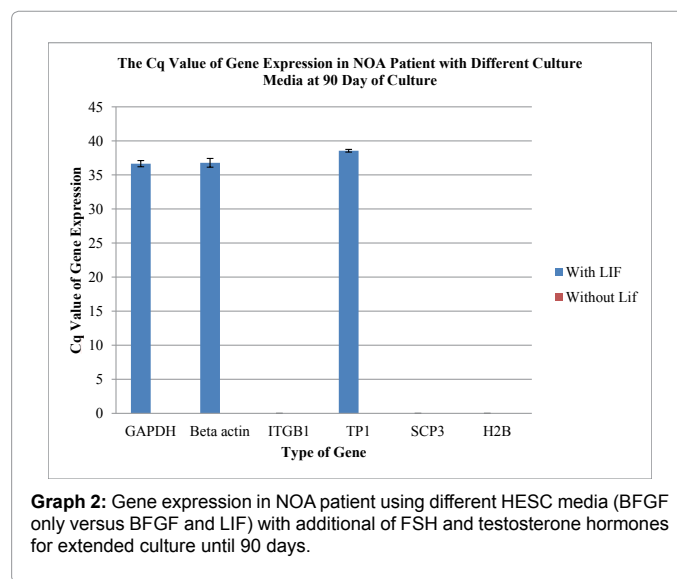
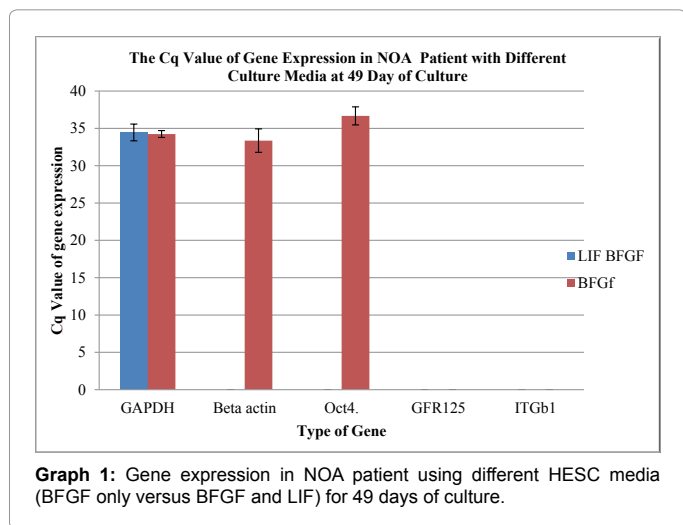


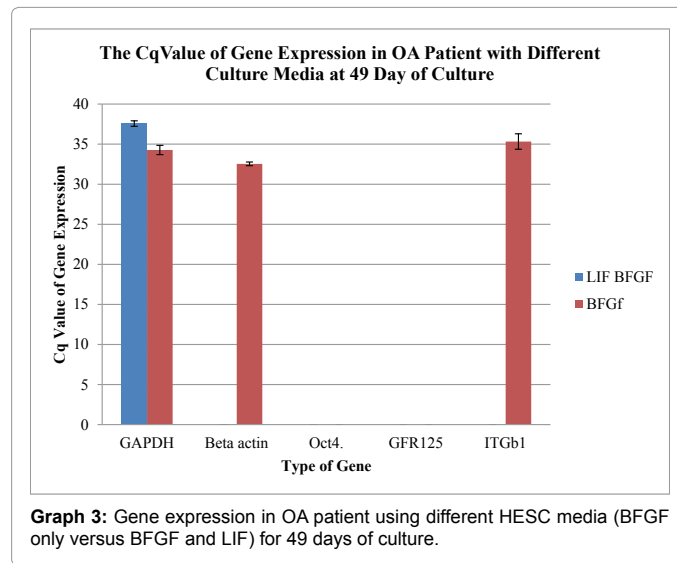
Figure 3: Propagation activities of SSC-like cell colonies *in vitro* culture in OA patient. **A:** Day 49, SSC-like cells cultured with HESC and BFGF (arrows); **B:** Day 49, SSC-like cells cultured with HESC, BFGF and LIF (arrows); **C:** Day 90; SSC-like cells cultured with IVF, FSH and testosterone (arrows); **D:** Day 90, SSC-like cells cultured with HESC, BFGF, LIF, FSH and testosterone (arrows).



Graph 2: Gene expression in NOA patient using different HESC media (BFGF only versus BFGF and LIF) with additional of FSH and testosterone hormones for extended culture until 90 days.



Graph 1: Gene expression in NOA patient using different HESC media (BFGF only versus BFGF and LIF) for 49 days of culture.



Graph 3: Gene expression in OA patient using different HESC media (BFGF only versus BFGF and LIF) for 49 days of culture.

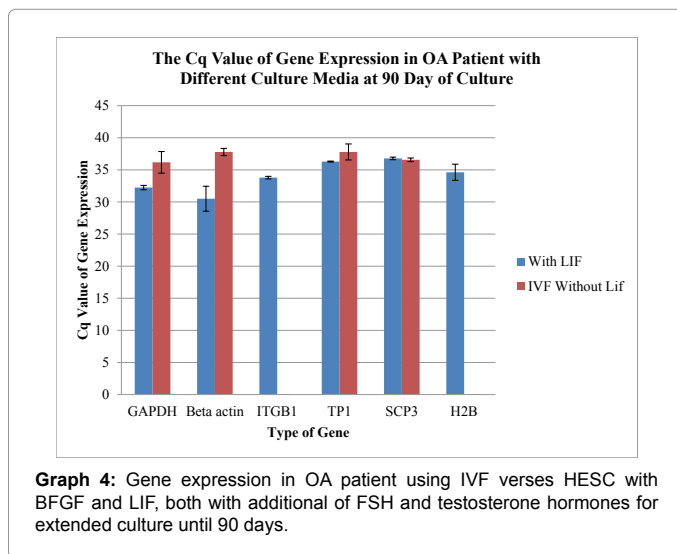
culture, only gene of TP1 was expressed in HESC media with BFGF and LIF (Graph 2). There was no gene expression in HESC media with BFGF.

As for in OA patient, only gene of ITGB1 was expressed in HESC media with BFGF only in 49 days of culture (Graph 3). After extended culture for 90 days, all genes (ITGB1, SCP3, H2B, TP1) were expressed in HESC media with BFGF, LIF, FSH and testosterone hormones. In IVF media with FSH and testosterone hormones, gene of SCP3 and TP1 were expressed. This result shown LIF plays an important role in long-term culture (90 days) than short culture (49 days) in both of the azoospermia patients (Graph 4).

Discussion

Study on isolation, culture and identification of undifferentiated SSC has allowed us to characterize the human testis phenotypically. SSCs are unique adult stem cells because they can undergo self-renewal and transmit genetic information to the next generation [9]. The biological study of human SSCs is a worthy research to understand the basic concept of stem cell regulation and therapy in male infertility problem such as azoospermic patient.

The establishment of specific culture system is important to maintain a specific type of self-renewal division [10]. The hESC culture media was used generally for the development of undifferentiated ESCs, but it was 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 SSCs. Knockout Serum Replacement (KSR) contains serum-free was added as a supplement to the culture condition. The main purpose of KSR is to provide hormones and growth factors to trigger cell replication [11]. These both reagents used together to produce less cells differentiation. The result of this study



is parallel with previous study reported that KSR is vital for testicular organ culture and the long-term culture of SSCs was supported by utilizing KSR [11,12].

Furthermore, the hypothesis of this study was to determine the combination of growth factor and HESC media can support the propagation activity of the cell populations. Based on SSCs morphology from day 1 up to day 49 of culture, the propagation activity of cells were gradually expanded from a single cell suspension attached to the plate until they aggregated and formed stable colonies. It showed that the propagation of "SSCs like cells" was positively seen. The result of this study suggested that BFGF alone in culture medium supported the propagation of the undifferentiated SSC-like cells in short-term culture but not in long-term culture. Oct4 was the only gene expressed in this short culture for NOA patient using HESC media with BFGF only. This short-term culture study shown undifferentiated spermatogonia with pluripotent characteristics occurred. As for OA patient using the same culture media, only ITGB1 gene was expressed, which represent SSC marker. Both of these genes were supported by previous study as stem cell marker [13].

According to previous study, combination of growth factor provides such as BFGF, LIF, EGF and GDNF were usually added into culture system for a long-term stabilization of SSCs propagation [9,10]. Our study proved that combination HESC media with BFGF, LIF and hormones (FSH and testosterone) have a tremendous effect on long-term culture (Day 90) with positive result in our gene expression analysis for both of the azoospermia patients. However, combination of BFGF and LIF has less effect on short-term culture (Day 49) compared to BFGF alone in HESC media for both of azoospermia cases (OA and NOA). LIF is to facilitate establishment of germ cell colonies but not increased the proliferation activity of SSC [14,15]. It is still yet to understand the function of LIF in long-term culture that helps in promoting the later stage of spermatogenesis process compared to BFGF alone in HESC media. FSH plays a role in mitosis process to form SSC and germ cell maturation where differentiating SSC into round spermatid through meiosis process. Testosterone potentiates FSH action and stimulates late spermatid differentiation [14]. In NOA patient, the SSC-like cells able to differentiate into spermatid with TP1 gene expression identified using HESC media with BFGF, LIF and

hormones. As for OA patient, all genes were expressed; SCP3, H2B and TP1, which represent advanced stages of spermatogenesis (meiotic spermatocytes and spermatid respectively). All the gene markers for meiotic spermatogenesis were proven by previous study [16]. Our study has shown positive result with the present of SSC colonies in OA patient with HESC and BFGF media for 49 days of culture. Therefore, this sample was change to IVF media for extended culture with combination of FSH and testosterone hormone (Day 90) as this media can initiate the growth of female germ cells. IVF media is well known as an embryonic media to initiate fertilization of oocyte and developing embryo growth. Our result shown SCP3 and TP1 genes were expressed which represent the later stage of spermatogenesis.

We had normalized the interest genes with housekeeping genes of GAPDH and beta actin. Beta actin is not particularly good as housekeeping gene for culture using LIF alone at 49 days of culture, it showed in OA and NOA sample. The additional LIF have been postulated to interfere with the expression of beta actin but with the present of BFGF only this interference had been resolved. Thus the database of specific housekeeping genes to be used for specific cells or tissue should be established since the cells have different gene expression patterns.

This study suggested that HESC media with BFGF could support for short-term culture of SSC-like cells. Extended culture with HESC media in combination of BFGF, LIF, FSH and testosterone hormones maintain differentiation of SSC-like cells into later stage of spermatogenesis. Oct4 and ITGB1 are SSC markers in spermatogenesis while SCP3, H2B and TP1 are genes that represent the later stage of spermatogenesis. Further research should be done to understand the biology and survival requirements for spermatogenesis cell culture in a short period of time. The function of LIF could be explored more in long-term culture and interference in gene housekeeping beta actin expression pattern.

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