Original Article

Multipotent SSEA-1 Positive Cells Population Differentiation into Primordial Germ Cells and Subsequently Progress into Oocyte-like Cells

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Abstract

Background: Stage-specific embryonic antigen- 1 (SSEA1) is a cell surface carbohydrate that its pattern expression is changed during induction of mouse embryonic stem cell differentiation. In this study, the spatial distribution of SSEA1 on primordial germ cells differentiation and subsequent progression into oocyte-like cells from mouse embryonic stem cells in vitro was evaluated.

Methods: Embryoid bodies from mouse embryonic stem cells were cultured for two days with 5 ng/mL BMP4. SSEA-1 positive and negative cells were separated using the MACS system and cultured separately in a conditioned medium consist of in vitro maturation medium diluted in DMEM [1:1] for 10 days. We assayed viability, colony formation and alkaline phosphatase activity (ALP) of sorted cells. Also, germ cell markers were analyzed by flow cytometry, Immunocytochemistry and RT-PCR.

Results: Viability percent SSEA-1 positive cells were more than SSEA-1 negative cells. SSEA-1 positive cells and SSEA-1 negative cells formed compact and flat colonies respectively. Unlike the SSEA-1 positive population, the SSEA-1 negative colonies showed a weak ALP activity. SSEA-1 positive cells expressed Oct4, Stella, Mvh, c-kit, Scp3, Desmin, GFAP and Albumin. Interestingly, SSEA-1 negative cells expressed Desmin and GFAP. The population of Mvh-positive cells in SSEA-1 positive was 17.74%. All specific oocyte mentioned genes were detected in the SSEA-1 positive. Also, oocyte specific proteins GDF9 and ZP3 were detected using Immunocytochemistry.

Conclusion: Our results suggest that conditioned medium provides a suitable niche to differentiation and progression putative primordial germ cells derived from the SSEA-1positive toward oocyte-like cells.

Keywords: BMP4, conditioned medium, embryoid bodies, SSEA-1

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Introduction

Embryonic stem cells (ESCs) can be induced to differentiate along various pathways, depending on appropriate culture conditions, readily committing to primordial germ cells (PGCs) and as well as other lineages.[1,2]

Some differentiation factors have been shown to induce PGCs differentiation of stem cells, including Bone Morphogenetic Proteins (BMPs) which is a member of transforming growth factor β (TGF-β) super family of intercellular signaling proteins and has a notable value in PGCs induction.[3] BMP4 is required for the generation of PGCs in the mouse embryo. At target cells, heteromeric complexes of BMP dimmers phosphorylate BMP receptors and activate signaling pathway, including Smads that regulate transcription of BMP target genes.

A general aspect of mouse ES cells during differentiation is a change in glycoproteins. The nature of cell-cell contacts suggest that the status of intercellular adhesion molecules on ES cells may alter upon differentiation.[4–7]

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Recently, several studies showed that some cell adhesion-related molecules (CAMs) were expressed at high levels on undifferentiated ES cells, but down-regulated soon after the induction of differentiation. These differences in patterns of expression suggest that CAMs may be part of the overall phenotype between differentiated and undifferentiated ES cells.[1,2]

Stage-specific embryonic antigen-1 (SSEA1), identified as the cell surface carbohydrate antigen Lewis, is a homophilic adhesion molecule and is capable of interaction with itself and other CAMs. Carbohydrate– carbohydrate interactions are important in specific recognition between cells,[8,9] especially during differentiation and embryogenesis.[8]

The expression of SSEA1 was observed in ES cells, but not in their differentiated derivatives.[9] Thus, SSEA1 can serve as an excellent cell surface marker to study ES cells differentiation.[10]

Although, the process of PGC development in vivo have been known up to now, but the patterns of PGC development in vitro is still poorly understood. The most robust marker for identifying the presence of germ cells in a population is expression of VASA. Recently, SSEA1 was reported as novel cell surface markers for identifying in vitro derived PGCs during ESC differentiation.[11] Since, expression patterns of SSEA1 occur in undifferentiated and differentiated ES cells.[12]

Therefore, it seems that the spatial distribution of SSEA1 was changed during induction of differentiation mouse m ES cells to PGCs.

There are many controversies surrounding SSEA1 marker.

Some reports consider SSEA1 as a PGCs marker, however the others consider it as a pluripotency marker. Some studies also showed that the addition of BMP4 could induce the expression of germ cell-specific markers.

In this study, BMP4 was rendered as induction medium and then the spatial distribution of SSEA1 on PGCs differentiation and subsequently differentiation into oocyte-like cells (OLCs) from mESC in vitro was examined. In addition, we investigated the pluripotency characteristic of SSEA1-positive) and negative (SSEA1+ve and –ve) cells.

Material and Methods

ESC culture

CGR8 mESC, established from Mus musculus strain 129 (a gift from Dr. M. Solimani), was maintained in the absence of feeder cells. The cell line was cultured on gelatin (0.1% Sigma) –coated 50-mL plastic flasks (Falcon, Becton Dickinson) in ES medium; DMEM (Gibco) supplemented with 15% fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Gibco), LIF (1,000 IU/mL; Chemicon, Boronia, Australia), 1% w/w non-essential amino acids (Gibco), 0.1 mM β- mercaptoethanol (Sigma) and 1% w/w penicillin/streptomycin (Gibco).[13]

Embryoid bodies

Embryoid bodies (EBs) were created by hanging drop (HD) method.[6] To induce the formation of EBs, ES cells were dissociated into single cells, re-suspended in the EB medium (ES medium without LIF) to a concentration of 2000 cells per 20 µL. Twenty micro liter drops of the suspension were put on the inner surface of a petri dish lid. The cells were incubated at 37 °C in 5 % CO_2 for 48 hours. Then, EBs were collected and transferred in individual wells of low-attachment plate with differentiation medium (DM) that consisting of EB medium with 5 ng/mL BMP4 for 2 days. BMP4 was reconstituted in 0.1% bovine serum albumin (BSA) in 4 Mm HCL, as per the manufacturer's instructions (R & D systems).

Magnetic cell sorting (macs) separation

SSEA-1 positive and negative cells were separated using the MACS system (Miltenyi Biotec; Bergisch Gladbach, Germany) according to manufacturer's instructions.[2] Briefly, the cells were resuspended in 0.5% BSA/PBS (Phosphate buffered saline) and incubated with anti-SSEA-1 antibody (10 µg/mL) for 20 minutes at 8ºC. Subsequently, the cells are magnetically labeled with rat anti-mouse IgM (Miltenyi Biotec) for 20 min at 8ºC. The cells were then washed three times with 0.5% BSA/PBS. For sorting and selection of SSEA1+ cells, labeled cells were loaded into a sterile LS column installed in a magnetic field. The column was rinsed with 0.5% BSA/PBS and the negative unlabeled cells were passed through and collected. Trapped cells were eluted after the removal of the column from the magnetic field and were collected by centrifugation.[14] The SSEA1+ve and SSEA1-ve cells were cultured separately in a conditioned medium (CM) for 10 days. Conditioned medium was modified by In Vitro Maturation medium (IVM medium) diluted in DMEM [1:1] (dIVM).

IVM medium

IVM medium was composed of MEMα medium (Invitrogen), supplemented with 1U/mL pregnant mares` serum gonadotropin (PMSG), 0.23 mM Sodium pyruvate, and 1mg/mL BSA.

MTT assay

The viability of isolated cells was carried out by methyl thiazolyl tetrazolium (MTT) in day 0, 2, 4, 6, 8 and 10. Briefly, 4.8×10^4 cells per 200 mL medium was seeded into each well of a 96-well micro liter plate for 24 hours at 37 °C in 5% CO2. Then, 50 µL of 1 mg/mL MTT solution (Sigma-Aldrich) constituted in PBS was added to each well and the cells were incubated for 4 hours at 37 ° C in the dark. After incubation, the media was removed and replaced with 50 µL of 100 % Dimethyl sulfoxide (DMSO), then placed on a shaker for 5 – 10 min to agitate and dissolve the formazan crystals.[15] Absorbance at 570 nm was measured in a Cytofluor 4000 plate reader (PerSeptive Biosystems, Framingham, Massachusetts, USA). All experiments were performed in three replicate wells.

Alkaline phosphatase (ALP) activity

For detection of ALP activity, the SSEA1+ve and -ve sorted cells were washed three times in PBS. The cells were fixed in a solution containing 2.5 mL citrate, 6.5 mL acetone and 0.6 mL formaldehyde in distilled water for 30 min at room temperature (RT). Then the fixed cells were incubated in a solution containing 0.5 mg/mL Fast Red Violet (Sigma) and 40 µL/mL α-naphthol phosphate (0.25% solution) for 30 min. The ALP activity of mentioned cells was observed using light microscopy.[14]

Immunocytochemistry

For immunocytochemistry, the SSEA1+ve and -ve sorted cells were washed with PBS at PH 7.4 and fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature (RT). The fixed cells were permeabilized with 0.2% Triton X-100 for 10 min at RT followed by three washes with PBS. To block unspecific binding of the antibody, the cells were incubated with 10 % goat serum for 30 min at RT. The cells were also incubated with primary antibodies GDF9 (Goat polyclonal IgG, Santacruze, SC- 12244) which diluted 1:1000 in TBST [Tris buffered saline (TBS), 1% BSA and 0.1% Tween-20 (all Sigma)] as well as ZP3 (Goat polyclonal IgG, Santacrouze, SC- 23717) which diluted 1:100 in TBST for overnight (O/N) at 4°C. The cells were washed three times with PBS. Further incubation with the appropriate secondary antibodies Phycoerythrin (PE) -conjugated Donkey polyclonal secondary antibody to Goat IgG (ab976, abcam system) and FITC- conjugated Donkey polyclonal secondary antibody to Goat IgG (ab975, Invitrogen) were performed which diluted 1:100 in TBST for 45 min at RT in dark. Then the cells were washed eight times in PBS. Nuclei were detected by 3,3'-diaminobenzidine (DAPI, sigma) staining. Images were captured with an Olympus phase contrast microscope (BX51, Olympus, Tokyo, Japan).[15–18]

Flow cytometry

The SSEA1+ve and -ve sorted cells were collected, then washed with 1% FCS in PBS and treated with trypsin/EDTA for 5 min to form single cells. For intracellular staining of Mvh protein, cells were fixed in 1% PFA for 10-15 min at 4°C for stabilizing proteins, followed by permeablizing of cells in 250 µL of detergent (Triton-X 100). Fixation/Permeablization procedures were performed on ice. Then, the cells were washed by adding 2 mL of PBS (containing 0.1% triton), centrifuged at 300g (2000 rpm) for 5 min. The cells were labeled with primary antibody Ddx4/Mvh (rabbit poly-

clonal IgG, ab13840, Abcam System, UK) for 30 min at 4°C in dark, then washed 3-times by centrifugation at 300g (2000 rpm) for 5 min and resuspended in ice cold PBS. The phycoerythrin (PE)-conjugated donkey polyclonal antibody to rabbit IgG (ab 7007, Abcam System, UK) was used as a secondary antibody and the cells were incubated for 30 min at 4°C. The incubation had to be done in dark, then washed 3-times by centrifugation at 300g (2000 rpm) for 5 min and resuspended in ice cold PBS.[18] Analysis was performed using a BD FACS Caliber (Becton Dickinson, San Jose, CA, USA) and FlowJo software (WinMDI 2.9, J. Trotter).

RNA extraction and PCR

Total RNA was extracted from SSEA1+ve and -ve sorted cells using the Qiazol lysis Reagent (Qiagen) according to the manufacture's instruction. Total RNA was quantified, and 5 µg was used for cDNA synthesis using random primers Fermentas under the standard conditions.[17,19–21] RT-PCR amplifications were conducted for 3 min at 95 °C (95 °C, 30 second; 60 °C, 45 second; and 72 °C, 45 second) for 40 cycles and 72 °C: 7 min for final extension. Primer sequences are as shown in table 1:

Data analysis

Quantitative data was expressed as a mean plus or minus SEM from at least three experiments. Student's t-test was used for statistical analysis with P values less than 0.05 which was significant.

Results

Our results showed that the viability percent SSEA1+ve cells were more than SSEA1-ve cells. The mean viability rates of SSEA1+ve cells gradually increased from D0 to D8, following with a significant decrease. Whereas, the mean viability rates of SSEA1-ve cells was low and gradually decreased (Figure 1).

The isolated cells were cultured in CM for 10 days, After 4 days; both isolated cells can form colonies, especially SSEA1-ve cells that subsequently formed flat and loosely colonies (Figure 2). The SSEA1+ve cells formed compact colonies. As shown in figure 2A, the SSEA1+ve single cells and SSEA1+ve colonies showed a strong ALP activity. Unlike the SSEA1+ve, the SSEA1-ve colonies showed weak ALP activity (Figure 2B).

In order to characterize the multipotency of the isolated cells, we performed molecular assay by RT-PCR. The SSEA1+ve cells expressed Oct4 or Pou5f1 (Pluripotency marker), Stella, Mvh, c-kit, Scp3 (Germ cell markers), Desmin (Mesodermal lineages), GFAP (Ectodermal lineages) and Albumin (Endodermal lineages). Interestingly, the SSEA1-ve cells expressed Mesodermal lineages Desmin as well as Ectodermal lineages GFAP (Figure 3).

Based on Mvh-positive cells, we assayed PGCs formation from isolated cells by flowcytometry. The population of Mvh-positive cells in SSEA1+ve cells was 17.74%, whereas it was approximately 4.89% in the SSEA1-ve cells (Figure 4).

From the oocyte-specific markers investigated, and SSEA1+ve cells expressed GDF9 and Figα. The marker ZP1 was expressed by SSEA1+ve cells and weakly expressed by SSEA1-ve cells (Figure 5). Next, immunocytochemistry assays was performed to distinguish the putative oocyte-like cells derived from SSEA1+ve cells, we observed GDF9 and ZP3 proteins (Figure 6). The oocyte-like cells were approximately 55 µm in size (Figure 6).

Table 1. Quantitative RT-PCR Primer Sequences

Gene	Primer (forward/reverse)	Significance
Oct4	5'- CTCGAACCACATCCTTCTCT -3' 5'- GTTCTCTTTGGAAAGGTGTTC -3'	Pluripotency marker
Stella	5'- TGAAGAGGACGCTTTGGA -3' 5'- CTTTCAGCACCGACA ACA -3'	Germ cell marker
Mvh	5'- CGGAGAGGAACCTGAAGC -3' 5'- CGCCAATATCTG ATGAAGC -3'	Germ cell marker
c-Kit	5'- CATGGCTGCATTCTGACAAATTCAC -3' 5'- CTCCATCGGTTACAAATACTGTAG -3'	Germ cell marker
Scp3	5'- TCAAAGCCAGTAACCAGA -3' 5'- TCGAACATTTGCCATCTC -3'	Meiotic marker
Desmin	5'- TCTCCCGTGTTCCCT -3' 5'- ATACGAGCTAGAGTGGCA -3'	Mesodermal marker
GFAP	5'- TGGATTTGGAGAGAAAGGTTGAAT -3' 5'- CGATAGTGGTTAGCTTCGTGTTTG -3'	Ectodermal marker
Albumin	5'- TCAACTGTCAGAGCAGAGAAGC -3' 5'- AGACTGCCTTGTGTGGAAGACT -3'	Endodermal marker
ZP1	5'- CCTCTCACCCTCTGTGGAACAG -3' 5'- GAGCATGTATCAGACCCAGAGG -3'	Oocyte-specific marker
Figα	5'- CCGTTTCTACCACAGAGCAGG -3' 5'- TTCTTCAAGCCACTCGCACA -3'	Oocyte-specific marker
GDF9	5'- CCAGCAGAAGTCACCTCTACAA -3' 5'- ACATGGCCTCCTTTACCACA -3'	Oocyte-specific marker
GAPDH	5'- ACCACAGTCCATGCCATAC -3' 5' - TCCACCACCCTGTTGCTGTA -3'	Internal Control

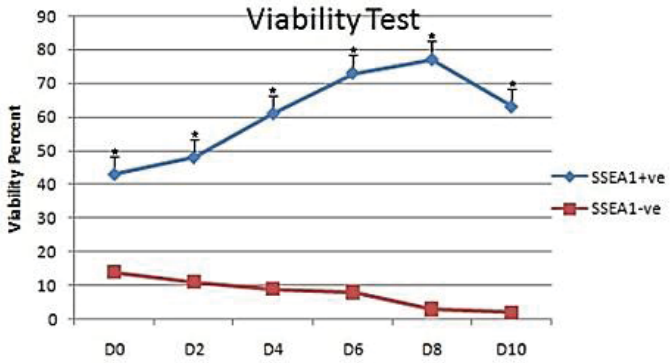

Figure 1. The comparison between the mean viability rates in SSEA1+ve and SSEA1-ve cells in days (D) D0 to D10. The significant differences among the mean percent of the viability were observed at D0, D2, D4, D6, D8 and D10. Asterisks represent significance ($P < 0.05$).

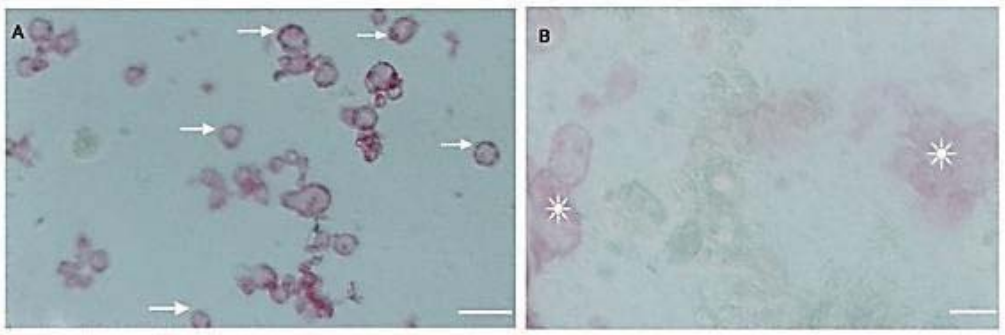

Figure 2. The isolated cells were stained by ALP; A) The SSEA1+ve cells were stained using ALP (Arrows show strong ALP activity), note some SSEA1+ve cells formed compact colonies; B) None of the SSEA1-ve cells were not stained by ALP; except that cells formed flat and loosely colonies (Asterisks) which are showed weak ALP activity. Scale bars represent 20 μm.

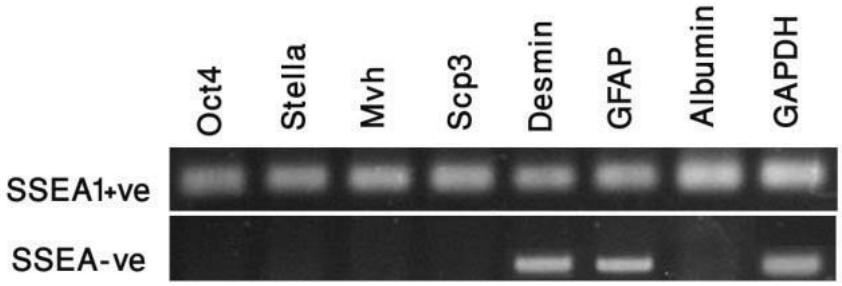

Figure 3. Reverse transcription–polymerase chain reaction (RT-PCR) analysis for SSEA1+ve and SSEA1-ve cells. GAPDH served as an internal mRNA control.

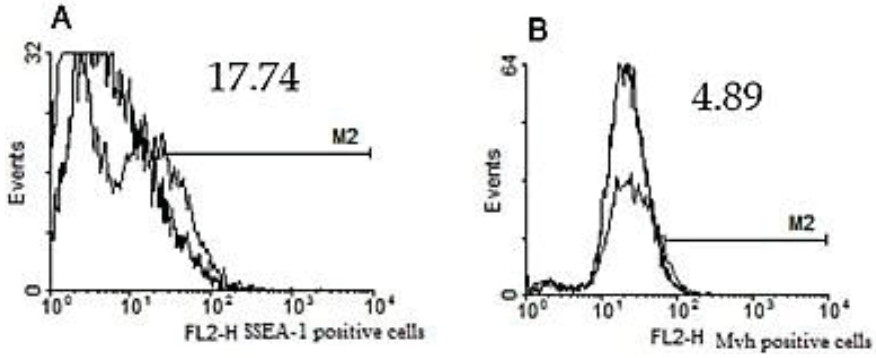

Figure 4. Analysis by flow cytometry indicated a small population of Mvh-positive cells in mESC; Proportion of Mvh-positive cells derived from SSEA1+ve cells (17.74%) and SSEA1-ve (4.89%).

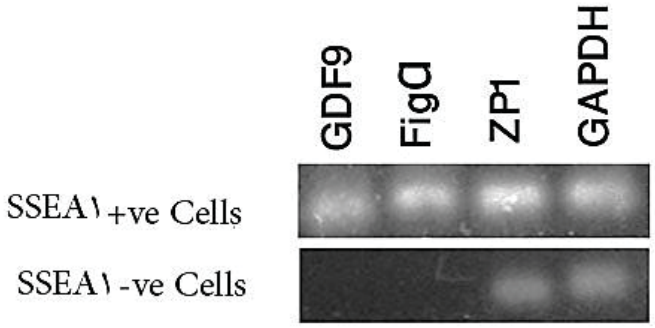

Figure 5. RT-PCR analysis for Non-sorted and SSEA1+ve cells. GAPDH served as an internal mRNA control.

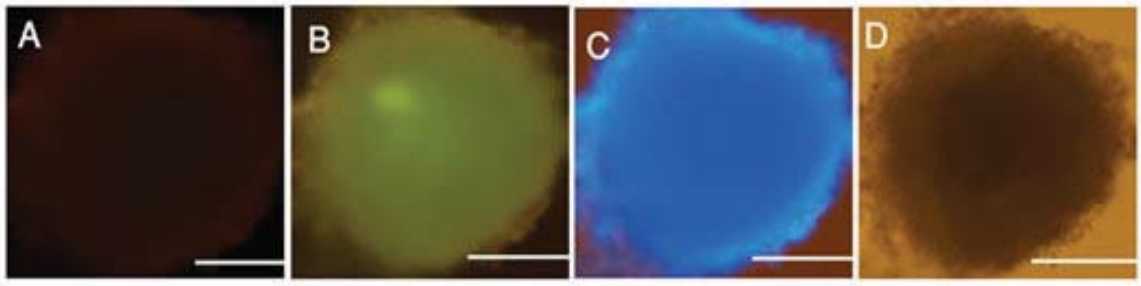

Figure 6. Expression of specific oocyte proteins was analyzed by Immunocytochemistry; A) GDF9 (red); B) ZP3 (green); C) Nuclei were stained by DAPI (blue) and D) Phase contrast photomicrograph of oocytes. Scale bars, approximately 20 µm.

Discussion

In the present study, we demonstrated that the SSEA1+ cells not only differentiated into three germ layers, but also differentiated into PGCs and subsequently to OLCs.

The condition medium described in our research showed that the viability percent was significantly higher in SSEA1+ cells than in SSEA1- cells. Previous studies, reported the effect of different growth factors on SSEA1+ cells proliferation and colony formation.[14,16,19] For example Bonnet, et al. first demonstrated the SSEA1+ cells and SSEA1- cells sorted from bone marrow mesenchymal stem cells with more than 90% purity. Secondly, they demonstrated that these cells were also essential to establish adherent cell cultures. Only the SSEA1+ cells are able to survive and propagate while, the SSEA1- cells did not go beyond the passage 0.[19]

Our results indicated that both the SSEA1+ve cells and SSEA1-ve cells were proliferated. These results are consistent with those of other studies and suggest that there are both the SSEA1+ve cells and SSEA1-ve cells in preimplantation mouse embryos and ESCs differentiated.[8] This heterogeneity occurs for other CAMs. It seems that differentiation fates may be randomly distributed in ES colonies as they are in preimplantation mouse embryos. The SSEA1 is a carbohydrate that interacts with itself, therefore carbohydrate–carbohydrate interactions are important during embryogenesis and colony formation that is known to be critical for proliferation as well as survival.[8–10] Another glycoprotein that was assayed, was ALP. According to our findings, the SSEA1+ve subpopulation formed compact colonies with strong ALP activities while SSEA1-ve subpopulation formed flat and loosely colonies with weak ALP activities that is consistent with Draper, et al. The distribution of SSEA1 expression alters that may be related to a transition in colony morphology.[20]

Following isolation of SSEA1+ve and SSEA1-ve cells, differentiation capacities of these cells were evaluated using RT-PCR. The SSEA1+ve cells were able to give raise not only to germ cell markers Stella, Mvh, c-kit, Scp3 but also to unconventional cells such as Mesodermal lineages, Desmin, Ectodermal lineages, GFAP, Endodermal lineages, and Albumin.[21,22]

This study produced results about the isolated cells, which corroborate the findings of previous work in this field. Characterization of the SSEA1+ve population derived from bone marrow mesenchymal stem cells showed that upon isolation, these cells differentiated into cells belonging to three germ layers.[16,19,23] These findings of the current study are consistent with those of Ratajczak, et al. who found the SSEA1+ve population derived from bone marrow mesenchymal stem cells differentiate into all three germ layers in an appropriate media [24, 25]. Similar results have obtained from the SSEA1+ve population showed a little expression of the neuronal progenitor marker genes GLI3 and PAX6. In addition, the SSEA1 is expressed by certain mesenchymal stem cell populations, like limbal stroma of the human eye.[20]

We further asked whether this SSEA1 isolated cells expressed main key transcription factor for the maintenance of the pluripotency.[11,26,27] The SSEA1+ve population was revealed the pluripotency-associated transcription factor octamer 4 (Oct-4), also known as POU5F. We could not observe this transcription factor in SSEA1-ve population. Therefore, it seems that the conditioned medium employed in our studies was suitable to maintain the expression of pluripotency marker Oct4. Based on the expression of Oct4, we believe that the SSEA1+ve cells maintain undifferentiated state. Pluripotency marker Oct4 is critical for maintenance of pluripotency during the early embryonic development, ES cells and for the viability of primordial germ cells.[26,28]

Most common genes are expressed during early germ cell development, which is applied to trace in vitro PGCs differentiation. Upon PGCs arrive the gonads, specific germ cell markers, including mouse vasa homologue (Mvh) gene, also known as

Ddx4, are transcribed. In mouse embryos, synaptonemal complex protein3 (SCP3) was detected during PGCs progress to meiosis.[5,6,29] Our data indicate that SSEA1+ve cells express markers that associated with PGCs differentiation. Current study showed that the SSEA1+ve population share many characteristics with ex vivo PGCs, like the expression of key genes Oct4, Stella and Mvh. In addition, the meiotic marker Scp3 were expressed in the SSEA1+ve population suggests that meiosis has been started.[3,30,31]

We further asked whether the SSEA1+ve cells can continuous expression of specific oocyte genes like GDF9, Figα and ZP1. We could not detect expression of these genes in the SSEA1-ve cells except ZP1 that was expressed weakly. However, all mentioned specific oocyte genes were detected in SSEA1+ve cells.

Our finding is consistent with the observations of Seki, et al. who indicated that the PGCs derived from SSEA1+ve cells share many characteristics, such as the expression of key genes and their cell cycle status. In addition, the putative PGCs population derived from the SSEA1+ve cells undergoes general epigenetic reprogramming changes, that indicates PGCs are in S-phase which suggests they may be preparing for entry into meiosis.[32]

In contrast to our method, Reijo Pera, et al. presented a novel method for the effective isolation of

PGCs and oocytes on the basis of properties of endogenous germ cell development. They used negative selection of SSEA1 to allow the isolation of Oct4-GFP+ and SSEA1- post-PGC-stage germ cells that exhibited properties of meiosis and maturation.[23]

Similar to endogenous fetal oocytes, ESC-derived oocytes also entered early stages of meiotic prophase I and expressed SCP1 and SCP3. However, SCP3 chromosomal alignment remained partial, and SCP1 was not elongated. Therefore, an in vitro block was occurred for meiotic progression.[33] Incomplete meiotic progression might be related to improper germ cell specification in vitro.[29,34] It seems additional factors that possibly related to PGCs development are required to overcome developmental block. A possible explanation is in vitro block to ovarian follicle formation. Granulosa cells surround meiotic oocytes to form follicles that are essential for functional oocyte. It seems that direct contact between granulosa cells and putative PGCs is required for signaling pathways of oocyte-independent development. Further investigations demonstrated that following transplantation, ESC-derived oocytes integrated into the ovarian niche, recruited somatic granulosa cells from the mouse ovary and directed follicle formation and development to the primary follicle stage.[13,23,28,34] As described in this research, we used dIVM medium that considered as an ovarian niche and might be suitable for oocyte differentiation.

According to Tingting Qing, et al. granulosa cells were effective in inducing the differentiation of ES cell-derived PGCs into oocyte-like cells through direct cell-to-cell contact. In addition, we used PMSG that mimics follicular stimulating hormone (FSH) and OLCs were observed approximately 55 µm in size. Therefore, PMSG triggers for in vitro growth oocytes. dIVM medium is similar to ovarian niche and contains all factors for derivation of OLCs from mESCs. It appeared that to be suitable for PGCs specification, proliferation, survival and differentiation into OLCs.[17,27,35]

In conclusion, these results suggest that CM (dIVM) provides a suitable niche to identify for the essential molecules for differentiation and progression putative oocytes from SSEA1+ve cells. Therefore, under this condition, cultivation of EBs for 2 days in suspension and subsequently for 10 days in CM, resulted in the formation of female gametes.

Acknowledgments

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