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Investigating The Effect Of Serum Concentration On The Expression Pattern Of Pluripotency And Lineage-Specific Markers In Mouse Embryoid Bodies Using Immunofluorescence

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الملخص:

تتمتع الخلايا الجذعية الجنينية (ESC) بقدرة التجديد الذاتي غير المحدود في المختبر وتتمايز إلى خلايا مكونة من جميع الطبقات الجرثومية الثلاث البدائية. الطابع المتجدد ESC يجعلها مصدرا جيدا لتطبيقات زرع الخلايا وهندسة الأنسجة. عندما يتم السماح ESC بالتمايز في مزرعة معلقة دون وجود عوامل مضادة للتمايز ، ينتج عنها تطور المجاميع متعددة الخلايا ثلاثية الأبعاد المعروفة باسم الأجسام الجنينية (EBs) . وهذه تمثل نموذجًا جيدًا لدراسة التطور الجنيني المبكر. يتأثر تمايز ESC داخل خلايا EBs بالعديد من العوامل الفيزيائية والكيميائية ، ومن هذه العوامل المصل في الوسط الغذائى الحاضن ، حيث أنه يحتوي على الهرمونات وعوامل النمو. في هذه الدراسة ، تم بحث تأثير تراكيز مختلفة للمصل على تمايز EB والفرق بين mRNA والتعبير البروتينى لعلامات النسب المحددة والاجسام الجنينية للفار اليوم الرابع من خلال استخدام صبغة المناعة. كما تم فحص الفرق في التعبير عن هذه العوامل بين ESC للفار غير المتمايزة و EBs للفار اليوم الرابع المتمايز ان التعبير البروتينى لـ Oct4 ، و Nanog ، و Bry ، وكانت خاضعة للتنظيم في EBS مطلي في مصل عالٍ ، وتشير النتائج الي تعبير Gata6 أكثر كان هذا متفقا مع مستوى تعبير mRNA والانتقال مع دليل على التنظيم الناقص للعلامات متعددة القدرات في الأديم الباطن الخارجي. بالإضافة إلى ذلك ، تم التعبير عن Oct4 بشكل إيجابي فىESC للفأرغير المتمايزة ، والتي تم تخفيضها بعد ذلك في EBs للفأر اليوم الرابع.

الكلمات المفتاحية:

الاجسام الجينية , الخلايا الجينية للفأرESC , التطور الجنيني المبكر.

Abstract

Embryonic stem cells (ESCs) have the ability of unlimited self-renewal in vitro and differentiate into cells composed of all three primitive germ layers. The renewable character of ESCs makes it a good source for cell transplantation and tissue engineering applications. ES cells when allowed to differentiate in suspention culture without anti-differentiation factors, 3D multicellular aggregates known as embryoid bodies (EBs) developed, these represent a good model for studying early embryonic development. ES cells differentiation within EBs is affected by many physical and chemical factors, one of these factors is the serum in the maintenance medium, as it contains hormones and growth factors. In this study, the influence of different serum concentrations on EB differentiation and the difference between the mRNA and protein expression of pluripotent and lineage specific markers of day 4 mouse EBs have been investigated by using immunostaining. The difference of expression of these factors between undifferentiated mouse ESCs and differentiated day 4 mouse EBs has been examined. The findings were the protein expression of Oct4, Nanog, and Bry were downregulated in EBs plated in high serum, while Gata6 expression was more this is consistent with expression level of mRNA and is in agreement with the evidence of down regulation of pluripotent markers in extraembryonic endoderm. In addition the Oct4 was positively expressed in undifferentiated mouse ESCs, which then reduced in differentiated day 4 mouse EBs.

Keywords: Embryoid body, Embryonic stem cells, pluripotent markers.

1. INTRODUCTION

Embryonic stem (ES) cells, which can be isolated from the inner cell mass of blastocyst stage of pre-implantation mouse and human embryos, have the ability to differentiate to form all three primitive germ layers and are thus termed `pluripotent' [1, 2, 3, 4]. ES cells were firstly isolated from mouse embryos in1981 [5], and from human embryos in 1998 [6].

Mouse ES cells can preserve their pluripotency and undergo unlimited self-renewal in vitro, particularly when maintained in the presence of leukemia inhibitory factor (LIF) and/or when cultured on feeder cell layers of mouse embryonic fibroblast, as they have the role of preventing their differentiation [7- 10]. In suspension culture, and in the absence of LIF and mouse embryonic fibroblasts, mouse ES cells spontaneously differentiate to form three- dimensional multicellular aggregates known as embryoid bodies (EBs), so-called because their development in culture resembles that of the peri-implantation mouse embryo [7- 9].

EBs represent a good model system for studying the early stages of embryonic development, as they can differentiate into derivatives of all the three germ layers [11]. The early stage of

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mouse EB development involves the cells aggregating in suspension in an E-cadherin-dependent process [12]. Which then change to three dimensional cystic bodies (Figure 1) that contain the three germ lineages [13]. The cells on the surface of the EBs differentiate to form primitive endoderm cells, which then undergo further differentiation to from parietal and visceral endoderm (VE) cells. The presence of bone morphogenic protein (BMP) inside the EBs is essential for the formation of the VE cells [14, 15]. The primitive endoderm deposits a basement membrane (BM) composed mainly of collagen type IV and laminin, that separates these cells from the remaining cells of the ICM, from which the germ layers are afterward derived [14,15]. In addition to its function as a physical barrier, the BM is essential for polarization of the epiblast cells to form a primitive ectoderm epithelium [14,15]. The cells located at the apical surface of this epithelium undergo programmed cell death, resulting in the formation of a proamnotic-like cavity within the EBs [3,14]. Following this, the primitive ectoderm generates definitive ectoderm, mesoderm and endoderm, though the process in EBs is more chaotic than the process of gastrulation which occurs in the embryo [14].

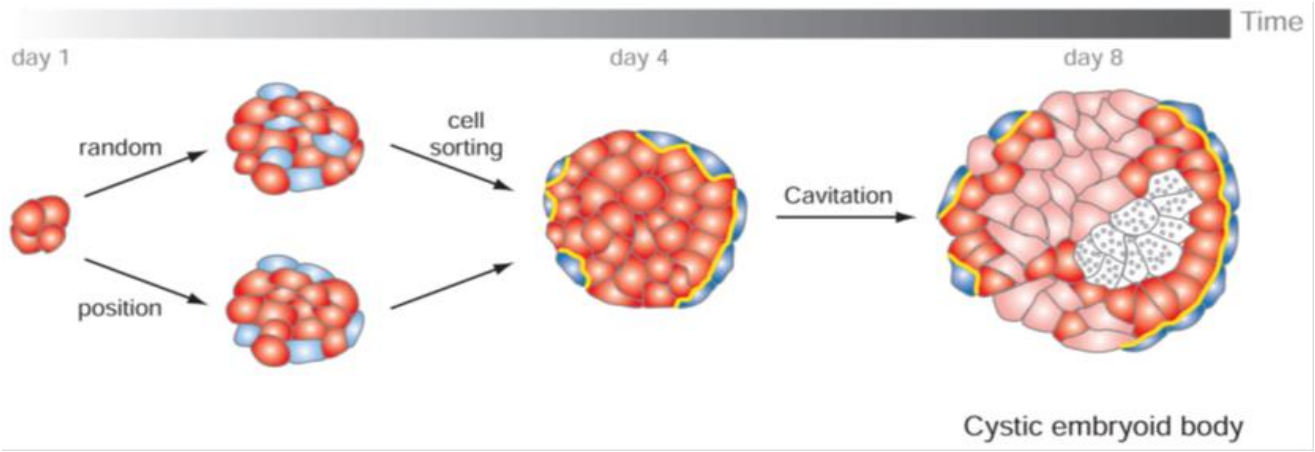


Figure. 1 Schematic diagram showing the developmental progression in the embryoid body which starts by the formation of an outer layer of extraembryonic endoderm cells (blue). Extraembryonic endoderm cells secrete ECM components to form a basement membrane which will contribute in the maturation of an epithelial epiblast and in the cavitation (grey dots indicate apoptotic cells). This developmental progress is heterogenous inside the embryoid body as it accompanied by cell differentiation (pink cells). Undifferentiated ES cells (red). Adapted from [16].

There is a thought that the pluripotency and self-renewal characteristics of ES cells rely on intrinsic, extrinsic factors, and many signaling pathways [17]. Recently, many studies have used microarray technology to examine the exact mechanism of self-renewal of ES cells, and these studies demonstrated that there were many transcriptional factors either expressed in ES cells but down-regulated with differentiation or expressed completely in ES cells in contrast to somatic cells [17]. One of the essential pluripotency transcription factors is Oct4, and also the group includes Nanog and Sox2 [17, 18]. These pluripotency transcription factors are necessary for the development of the mouse embryo during the early preimplantation stage; that is why they are highly expressed at this period [18,19]. Throughout the expansion of the blastocyst, these factors are expressed more in the inner cell mass and downregulated in the trophoectoderm [18]. Subsequently, the pluripotent markers are downregulated in the developing primitive endoderm [18].

While at the postimplantation stage, the expression of the pluripotent transcription factors are high in the epiblast of the egg cylinder stage and decreased in gastrulation, afterwards, during somite formation, their expression is confined to primordial germ cells and absent from all the parts of the embryo [18]. The pluripotency transcription factors positively regulate their own expression, and they also suppress the expression of genes required for cell differentiation [16, 18].

Throughout the developmental progression of EBs many transcriptional factors are expressed, which demonstrate their ability to generate cells from all three germ layers; for example, the phenotypic markers of endoderm [Foxa2, Sox17, GATA 4/6, and α-fetoprotein], mesoderm [Brachyury-T (Bry), Msp 1/2, Is1-1, and Odd-skipped-related 1 (Osr1)], and ectoderm [Sox1, Nestin, Paired box protein 6 (Pax6), and GFAP] [3]. Previous research using gene targeting has established that the transcription factor Gata6 is essential for extraembryonic endodermal cell development in vivo and in vitro [2,10]. Also these studies revealed that during the early blastocyst stage a number of cells in the ICM show expression of GATA6, subsequently, the expression becomes higher and restricted to the primitive endoderm cells [10]. The mesodermal transcription factor, Brachyury (Bry), is expressed only in the primitive streak, tailbud and notochord of the early mouse embryo [20].

One of the essential elements of mouse EB growth medium is serum as it contains necessary nutrients and hormones that can modulate the physiochemical properties of the medium [21]. It has been reported that by modifying one of the constituents of the culturing medium for example the restriction of the nutrient, reduction in serum concentration, and growth factors addition can affect the expression of some genes and cells proliferation [11]. As one study revealed that using lower serum concentration culturing medium can induce the expression of transcription factors responsible for the regulation of the differentiation of foregut ES cells to endocrine cells [22].

In my previous project, the impact of different serum concentrations on EB differentiation and the changes in gene expression of specific transcription factors was examined. In the current study the aim is to examine if there is a difference between the mRNA and protein expression of pluripotent and lineage specific markers of day 4 mouse EBs by using immunostaining. secondly second aim was, to investigate the difference of expression of these factors between undifferentiated mouse ES cells and differentiated day 4 mouse EBs.

2. METHODOLOGY:

The R1 mouse embryonic stem (mES) cell line and the STO (SIM (Sandos inbred mouse) 6-thioguanine resistant, ouabain resistant) mouse embryonic fibroblast cell line were a gift from Dr Neil Smyth, University of Southampton, UK. The E14-Bra-GFP mES cell line were a gift from DR. Georges Lacaud, Paterson Institute, Manchester.

STO cell culture

The STO cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Sigma) and 2 mM L-glutamine (Sigma). The cells were maintained in 10 cm gelatinized tissue culture dishes in a humidified atmosphere containing 5% CO2 at 37°C. The STO cells were sub cultured when necessary where the cells were washed with phosphate buffered saline (PBS; Sigma), dissociated using 1x trypsin/EDTA (Sigma) for 3-5 minute and split 1:10

by replating into 10 cm gelatinized tissue culture dishes and placed in the incubator. Passages 17-20 of STO cells were used for current experiments.

Preparation of feeder cell layers

The feeder layers for mES cells were prepared from STO cells that were treated with 20µg/ml of mitomycin C (Sigma) and replaced in the incubator for 2.5 h to arrest cells division. Following this, STO cells were washed with PBS, dissociated using 1x trypsin/EDTAfor 3-5 minute and split 1:10 by replating into 3.5cm gelatinized tissue culture dishes and incubated overnight.

Embryonic stem cell culture

The mES R1 and E14-Bra-GFP (passage18) cells were maintained on STO feeder cell layers in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine,1/100 non-essential amino acids (Sigma), 0.1 mM 2 mercaptoethanol (Sigma), 1,000 units/ml leukemia inhibitory factor (LIF; Millipore) and 1 mM sodium pyruvate. Cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C. In this experiment, mES (R1) cells were used between passages 14 and 20. Mouse ES (R1) cells were sub cultured every 2 days; this was done by washing the cells with PBS, dissociating using 1x trypsin/EDTA for 3-5 minute and splitting 1:3 by replating on the top of previously prepared STO feeder layers, then placed in 37°C 5% CO2 humidified incubator.

Embryoid body (EB) formation

For EB formation, mES cells were dissociated using 1x trypsin/EDTA and incubated for 3-5 minute. The dissociated cells were collected in EB medium (DMEM) supplemented with 1/100 non essential amino acids, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine and either 0%, 7.5% or 15% (vol/vol) FBS and plated in 10 cm bacteriological petri dishes at a density of 2.5×104 cells/ml. The medium containing the appropriate concentration of FBS was changed every 3 days.

To prepare frozen sections, EBs were collected at day 4 and fixed with 4% paraformaldehyde (PFA) and incubated at room temperature for 10 minutes. EBs then washed three times with PBS and stored in it at 4°C for up to 1week. Afterward, EBs were soaked overnight at 4°C in 15% sucrose to prevent ice crystals formation over them. Then sucrose solution were aspirated and 7.5% (wt/vol) of molten gelatin were added and incubated for 30-60 minutes in water bath at 37°C. Following this, EBs were aspirated within a droplet of gelatin and transferred onto a weight boat filled with molten gelatin, then the gelatin droplets that were set they were cut out of weighing boot and mounted with cryomountant (OCT) on cork disk and transferred to box with dry ice, when the samples had turned white they were wrapped in foil and stored at -20°C. The samples were cut at 10µm by using cryostat set to -20°C.

Immunostaining of EB sections

For immunofluorescence detection, EB sections were placed in a Coplin jar filled with PBS and incubated in water bath at 37°C for 20 minutes in order to melt off the gelatin. Then the blocking solution of 10% (vol/vol) bovine serum (Sigma) were applied to the sections and incubated in humidified chamber at room temperature for nearly 1 h. The primary antibodies used were anti-mouse laminin that recognizes all three subunits of Laminin-111 (15), anti-mouse Oct4, anti- rabbit Nanog, anti-rabbit Gata6, and anti-goat Brachyury (Table 1). In order to recognize unspecific binding of secondary antibodies controls should be included where primary antibodies were not added to primary antibody solution. Then the sections were incubated with the primary antibody solution in a humidified chamber overnight at 4°C. After that the sections were washed three times in PBS.

qFor secondary antibodies chick anti-rabbit Alexa fluor 488 was used for detection of the anti-laminin antibody, anti- rabbit Nanog, and anti-rabbit Gata6. While goat anti-mouse IgG2b Alexa fluor 594 for detection of the anti-Oct4 antibody and donkey anti-goat Alexa fluor 488 for detection of anti-goat Brachyury (Table 1). Secondary antibody solution were applied to the sections at room temperature for 2h, then the sections were washed three times in PBS.

After that DAPI with final concentration of 1:50,000 was applied to the sections for 5-10 minutes then washed with PBS once, then the sections were mounted in fluorescent mounting medium (Dako) and covered with cover slips. Sections were visualized with a Leica fluorescence microscope. Images were taken with same exposure and were processed similarly.

Table 1. List of antibodies used in this study

Antibody	Dilution	Source	Catalogue number
Anti-mouse laminin	1:500	Sigma-Aldrish	L9393
Anti-mouse Oct4	1:500	Santa Cruz Biotecnology	Sc-9081
Anti- rabbit Nanog	1:250	Cell Signaling Technology	D73G4
Anti-goat Brachyury	1:100	Santa Cruz Biotecnology	H2807
Anti-rabbit Gata6	1:250	Santa Cruz Biotecnology	I1503
Chick anti- rabbit Alexa fluor 488	1:1000	Invitrogen	A-21441
Goat anti- mouse IgG2b Alexa fluor 594	1:1000	Invitrogen	A-21145
Donkey anti-goat Alexa fluor 488	1:1000	Invitrogen	A-11055

Immunostaining of mES cell

In order to prepare E14-Bra-GFP mES cells for immunostaining, the cells were dissociated using 1x trypsin/EDTA. Cells were re-suspended with 15% (vol/vol) FBS DMEM medium, then the feeder layer depletion was carried out by culturing the cells twice on gelatinized tissue culture dish for 20 minutes in ES medium at 37°C and 5% CO2. The cells were then counted and 6x104 cell/ml were seeded in each chamber of the slide. For fixation of ES cells the medium were aspirated and 0.5 ml of 4% of PFA were added to each chamber, then incubated at room temperature for 5 minutes. Afterward, PFA were aspirated and washed three times in PBS. The blocking solution of 10% (vol/vol) bovine serum and 0.1%(vol/vol) Triton X-100 (Sigma) were applied to the cells and incubated at room temperature for 40-60minutes. The Triton was used in each solution applied to ES cells to permeabilize the cells so that the antibodies can access the antigen. Then the blocking solution were aspirated and primary antibody solution which prepared from 0.1%(vol/vol) Triton X-100, 1% bovine serum in PBS, and appropriate primary antibody with appropriate dilution were applied to the cells. The list of primary antibodies and used dilution in table 1. For the recognition of nonspecific binding of secondary antibodies controls should be included where primary antibodies were not added to primary antibody solution. Then the cells were incubated with the primary antibody solution in a humidified chamber overnight at 4°C. Following this, the cells were washed three times in PBS. The secondary antibody solution which consist of 0.1%(vol/vol) Triton X-100, 1%(vol/vol) of bovine serum in PBS, and secondary antibody with appropriate dilution (Table 1) were applied to the cells at room temperature for 2h, then the cells were washed three times in PBS. The counter staining of the cells was done by using DAPI with final concentration of 1:50,000 for 5-10 minutes then washed with PBS once, then the cover slip from the bottom of the chamber slide was removed and the glass slide was mounted in fluorescent mounting medium (Dako) and covered with cover slips. Samples were analyzed with a Leica fluorescence microscope. Images were taken with same exposure and were processed similarly.

3. DATA DESCRIPTION AND RESULTS DISCUSSION:

Morphological changes of differentiating EBs in different serum concentrations

In order to examine the influence of serum concentration on the process of cell differentiation within the mouse EBs, the EBs were plated in medium with 7.5% or 15% serum. Generally, the round aggregate structures of the EBs which formed from suspended mES cells, appeared within 24 hours and they were similar in shape despite the serum concentration differences. At day 4 the EBs plated in 7.5% serum differentiate to form thin layer of BM and single layer of PE. While those plated in 15% serum appeared with advances in the differentiation stage, as the BM presented as thick dark line with columnar visceral endoderm layer (Figure 2).

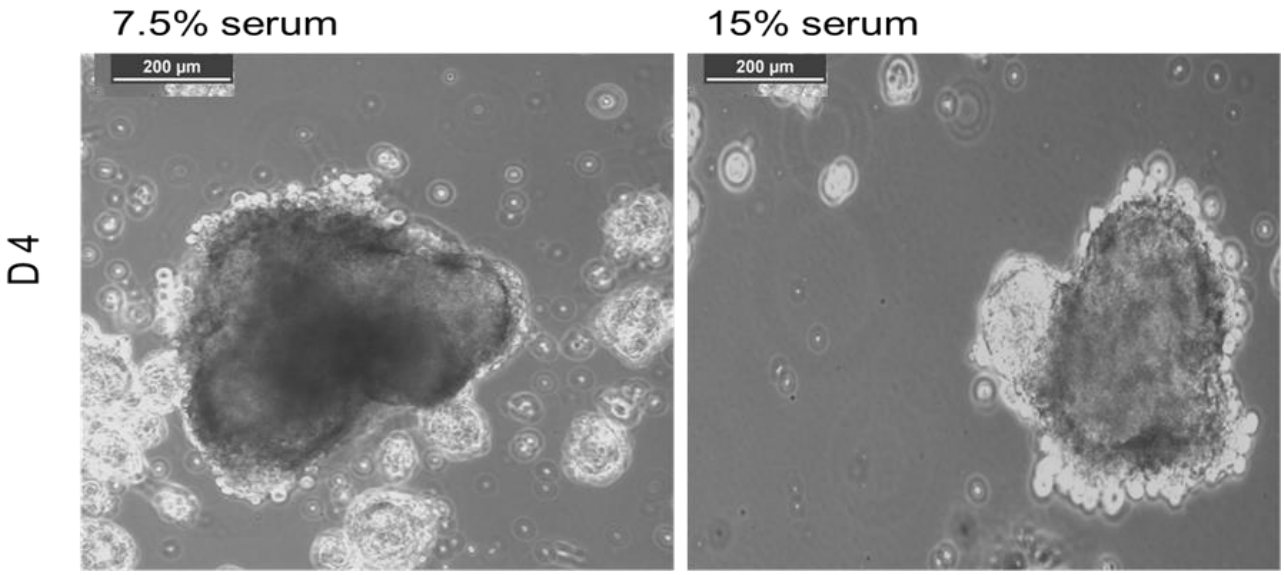


Figure.2. The morphology of developing day 4 EBs plated in different serum concentrations. EBs formed in lower serum concentration showed moderate differentiation with BM and single layer of PE. EBs formed in higher serum concentration appeared with advances in the differentiation as the BM appeared as thick dark line and the appearance of columnar visceral endoderm. Scale bars 200µm.

Immunofluorescence staining analysis of EBs plated in different serum concentrations

To establish the influence of serum concentration on the expression pattern of pluripotency and lineage-specific markers in mouse EB, immunofluorescence staining analysis was used. EBs were plated at two different serum concentrations (7.5%, and 15%). Day 4 EBs were stained with primary antibody solution contain antibodies against pluripotent markers, Oct4 and Nanog; the endodermal marker,Gata6; mesodermal markers, Bry and basement membrane marker, laminin. The fluorescent microscope is used to visualize the stained and unstained (controls) EBs, and they were photographed using a Leica camera.

The expression of Oct4 and Nanog throughout EB development

During the normal development of the mouse embryo the expression of Oct4 and Nanog are downregulated in the extraembryonic endoderm (18).

In figure 3 are shown day 4 EBs plated in 7.5% serum, the cells on the inside do not appear to express neither Oct4 nor Nanog. While those plated in 15% serum the cells on the outside loss the expression of the markers. With a quantitave analysis of positive expression of pluripotency markers in day 4 EBs revealed that the expression was more higher in low serum concentration (Figure 5). These results consistent with the previous project findings as the mRNA of the pluripotent markers level was higher in 7.5% serum compared to 15% serum.

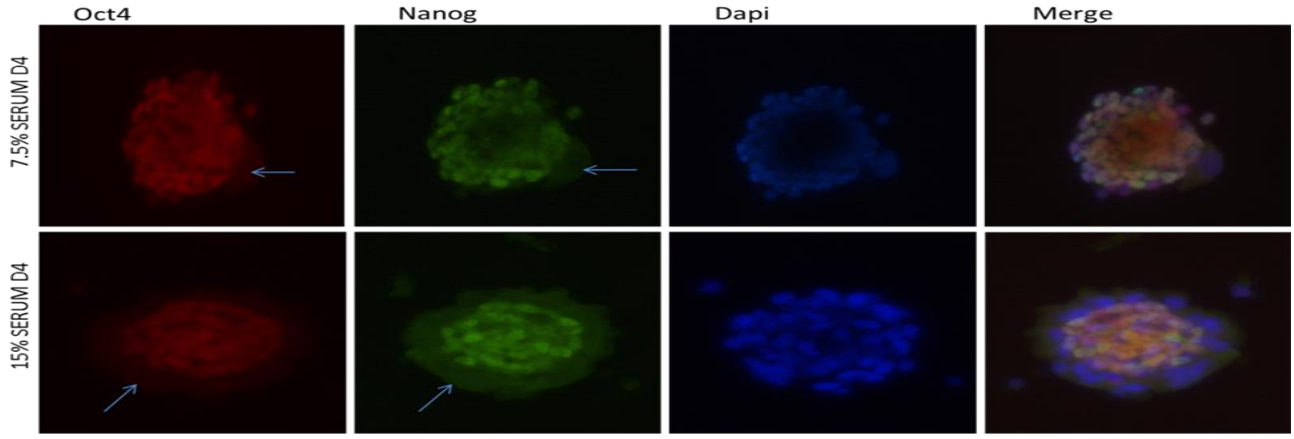


Figure.3. Immunostaining pluripotency markers Oct4 in day 4 EBs formed in different serum concentration. Green indicates positive staining for Nanog, red indicates positive expression of Oct4, blue indicates DAPI for nuclear staining and merge indicates the fusion of three colors by image J programme. In 7.5% serum the expression of Oct4 and Nanog downregulated as some cells inside and outside the EBs loss their expression (arrow). In 15% serum the loss of expression is more and form a big patch surround the hall EBs (arrows). Scale bars 50µm.

The expression of Bry and Gata-6 throughout EB development

Bry is firstly expressed in the posterior part of the epiblast, after that in nascent mesoderm of the primitive streak (18). In day 4 EBs there was expression of Bry in higher serum, which demonstrated as few numbers of scattered Bry positive cells in the interior of EBs. In contrast to lower serum which didnot reveal any Bry positive cells (Figure 4). This is similar findings to previous project.

The endodermal marker Gata6 was positively expressed in day 4 EBs regardless of the serum concentration difference. But the expression was more in 15% serum which indicates the acceleration in differentiation process took place in higher serum concentration (Figure 4, and 5). This findings were opposite to previous work.

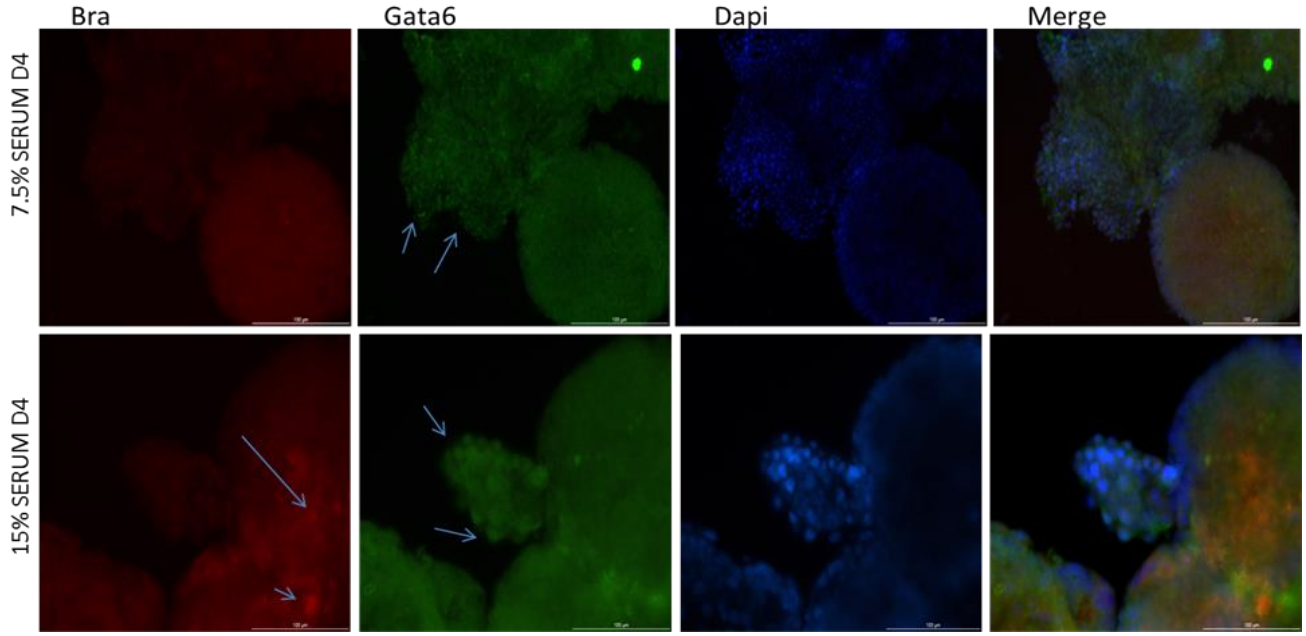


Figure. 4. Immunostaining of mesodermal marker Bry (red) and Gata6 (green) in day 4 EBs formed in different serum. Positive expression of Bry in the interior of EBs plated in high serum (arrows). Gata6 positively expressed in low and high serum but more obvious in high serum (arrows). Scale bars 50µm.

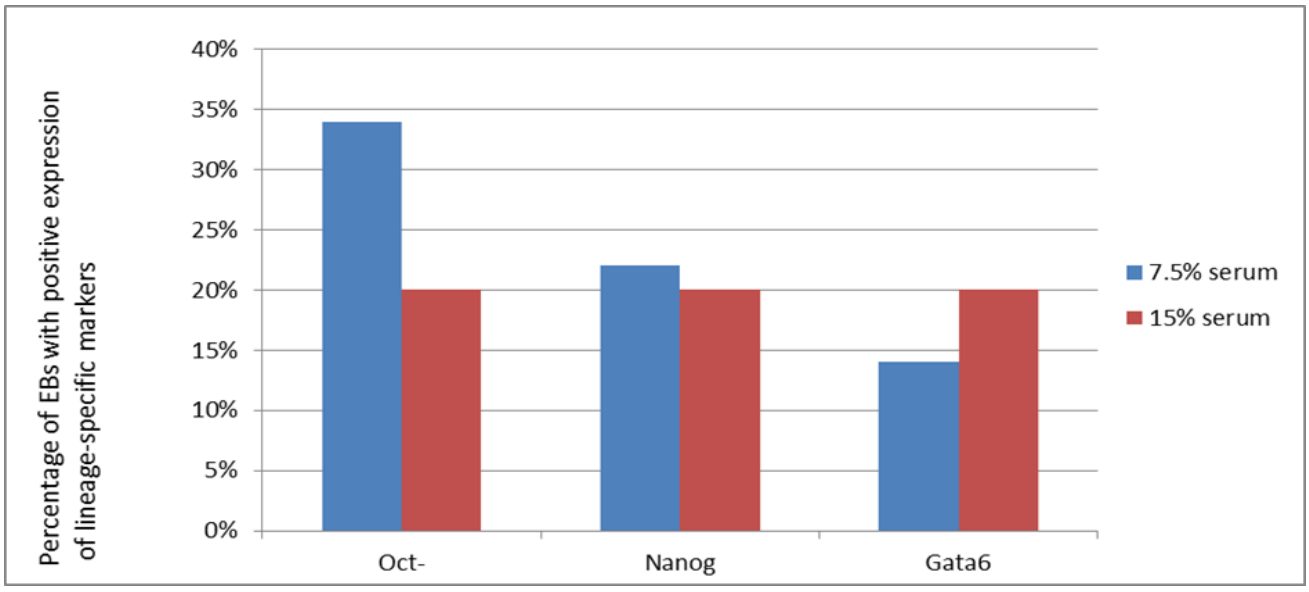


Figure.5. Bar charts represented percentage of day 4 EBs plated in different serum with positive expression of pluripotency transcription factors (Oct4 and Nanog) and endodermal marker (Gata6). The expression of Oct4 (41 EBs positive from 119) and Nanog (26 EBs positive from 119) was higher in 7.5% serum. The expression of Gata6 was more in 15% serum (39 EBs positive from 192). The immunostaining were carried out on three biological replicates and the result indicated as percentage of the average numbers.

BM formation in EB

BM formation in EBs have an essential role in cell differentiation and specialization in EBs (14). In current research immunostaining of laminin was used as a sign for BM formation. Figure 6 revealed that in both 7.5% and 15% serum the EBs develop well delineated BM surrounding most of the circumference.

Immunofluorescence staining analysis of mES cells

In order to study the difference of expression of pluripotent, endodermal, and mesodermal markers, between undifferentiated mouse ES cells and differentiated day 4 EBs, immunostaining for those markers were performed to E14-Bra-GFP mES cell line. The results were the ES cells positively express Oct4 (figure 7A), and the expression of Gata6 was positive and restricted to small clumps of cells (figure 7B). Unfortunately, the Bry antibody did not work. However, the immunostaining of laminin was done revealing the localization of this protein inside the cell and appeared as a clusters.

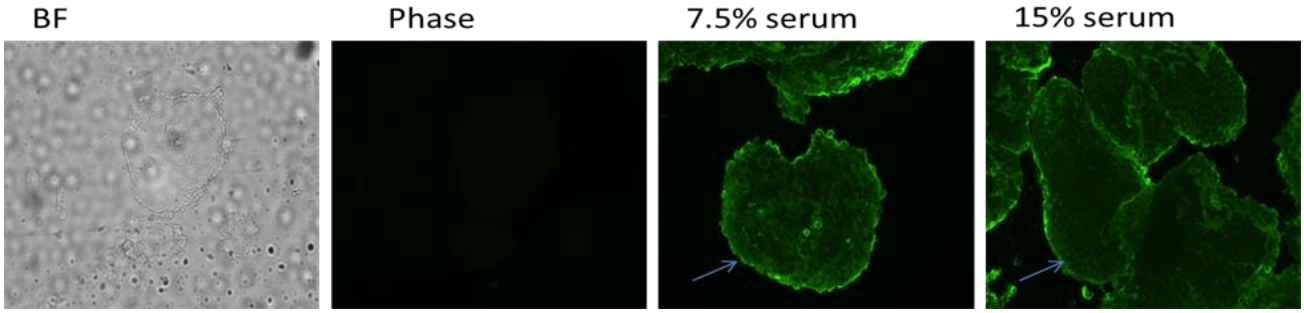


Figure. 6. Demonstrated bright field and phase contrast of no primary antibody controls. Also positive expression of laminin with well-defined BM in 7.5% and 15% serum concentration in day 4 EBs. Scale bars 100µm.

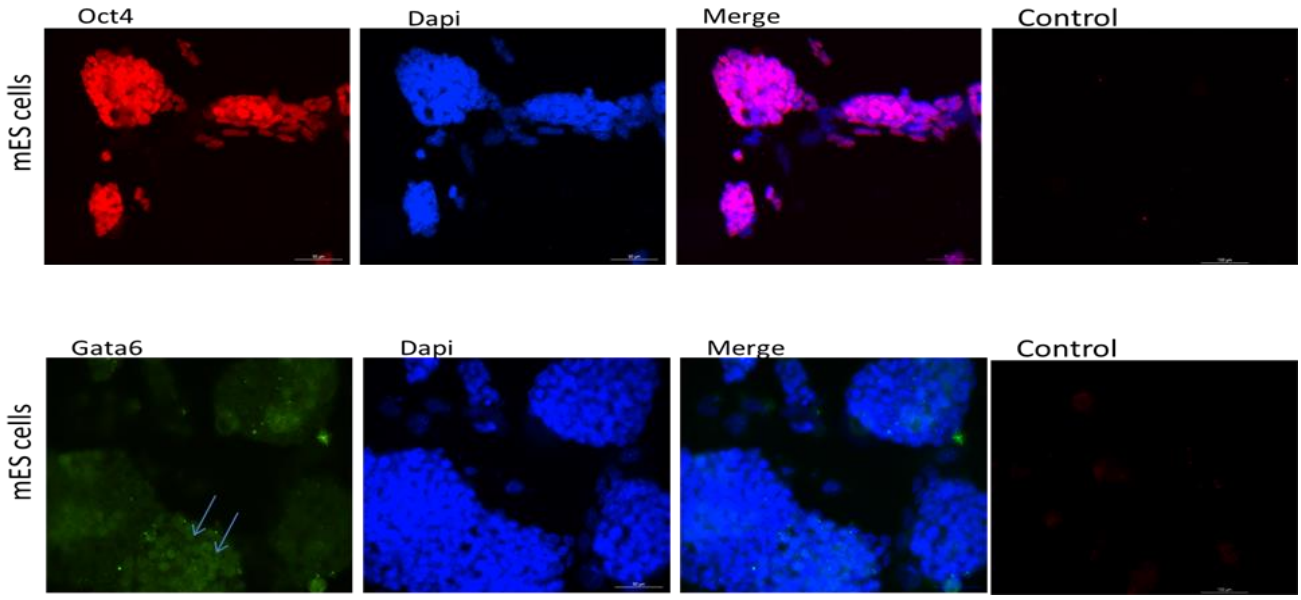


Figure. 7. Expression of Oct4 and Gata6. A. positive expression of Oct4 (red nuclei). B. Gata6 expression (green nuclei). Scale bars 50µm.

4. DISCUSSION:

Beside the therapeutic use of stem cells in degenerative diseases and tissue damage, stem cells are also have a wide applications in developmental biology research [2]. There were variety of in viro differentiation methods that allowed studying of ES cells derived from human and mouse embryos, one of them is growing ES cells in suspension to generate embryoid bodies [2]. Regardless of extensive use of EBs as the first step in the differentiation of pluripotent stem cells [18], there is little known about the undetermined factors in the serum that can influence EB development and differentiation [11].

Several previous researches on mouse EBs have investigated the influence of alteration of the constituents of culturing medium by adjusting serum concentration or adding growth factors on the differentiation progress [14]. However, these studies concentrate either on a particular cell type as cardiomyocytes [23], or the expression of definite transcription factors, as the relation of Gata4 over expression and the stimulation of cardiogenesis [24].

In this project the difference between the mRNA and protein expression of pluripotent and lineage specific markers of day 4 mouse EBs have been investigated by immunostaining, and how they influenced by different serum concentration (7.5% and 15%). Also the difference of expression of these factors between undifferentiated mouse ES cells and differentiated day 4 mouse EBs have been tested.

Our results revealed that the expression of pluripotent markers (Oct4 and Nanog) and mesodermal marker (Bry) are downregulated in high serum concentration while the endodermal marker (Gata6) is up regulated, this is consistent with the evidence that the loss of Oct4 during the development of murine EBs is essential for the formation of primitive endoderm and elevated level is associated with determination of cells to become mesoderm [18]. Moreover these findings indicate that the serum may contain specific factors that can accelerate the differentiation process in mouse EBs. The results of protein expression of those markers were similar to that were done on the mRNA in previous project [18] apart from Gata6 which was highly expressed in high serum concentration. Furthermore, the results demonstrated that the Oct4 was positively expressed in undifferentiated mouse ES cells, which then reduced in differentiated day 4 EBs. For Gata6 few cells were stained positively

This study is carried out on fixed age of EBs and specific markers were examined. Future work should include other types of germ lineage markers and the pattern of their expression over an extended period of EBs culturing.

5. CONCLUSION:

Overall, the results of immunostaining of pluripotent and some of lineage-specific markers is compatible with the results of RT-PCR analysis suggesting that the pluripotent markers have a dual role in regulating their own expression and other transcriptional factors, and the expression level of those markers can be affected by serum differences.

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