Video Article

Differentiation of Newborn Mouse Skin Derived Stem Cells into Germ-like Cells *In vitro*

Paul William Dyce[1,2]

[1]Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, The University of Western Ontario

[2]Children's Health Research Institute

Correspondence to: Paul William Dyce at Paul.Dyce@schulich.uwo.ca

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Abstract

Studying germ cell formation and differentiation has traditionally been very difficult due to low cell numbers and their location deep within developing embryos. The availability of a "closed" *in vitro* based system could prove invaluable for our understanding of gametogenesis. The formation of oocyte-like cells (OLCs) from somatic stem cells, isolated from newborn mouse skin, has been demonstrated and can be visualized in this video protocol. The resulting OLCs express various markers consistent with oocytes such as *Oct4 , Vasa , Bmp15*, and *Scp3*. However, they remain unable to undergo maturation or fertilization due to a failure to complete meiosis. This protocol will provide a system that is useful for studying the early stage formation and differentiation of germ cells into more mature gametes. During early differentiation the number of cells expressing Oct4 (potential germ-like cells) reaches ~5%, however currently the formation of OLCs remains relatively inefficient. The protocol is relatively straight forward though special care should be taken to ensure the starting cell population is healthy and at an early passage.

Video Link

The video component of this article can be found at http://www.jove.com/video/50486/

Introduction

During early embryogenesis, gametogenesis occurs through a series of stages including primordial germ cell (PGC) formation, migration, and finally colonization of the gonadal ridges. During this time PGCs undergo proliferation and differentiation into increasingly more mature gametes [1]. The fact that PGCs migrate from the base of the allantois into the embryo hindgut and finally along the dorsal wall eventually colonizing the gonadal ridges makes them exceedingly difficult to study [2]. Despite advances in the field, studies attempting to understand PGC formation and differentiation have been impeded by their limited number, location, and migratory nature [3,4].

In recent years embryonic stem cells have been shown to have the potential to form germ cells *in vitro* [5,6]. Similarly, several somatic stem cells have also been shown to have the potential to form germ cells following *in vitro* culturing [7-11]. Recently stem cells isolated from newborn mouse skin were differentiated *in vitro* into germ-like cells and early stage oocytes [12]. During differentiation the subset of the stem cells expressing Oct4 increased and structures resembling cumulus-oocyte complexes were formed. The oocyte-like cells (OLCs) can be picked from the cultures and compared to natural oocytes. Using this culture method OLCs measuring 40-45 µm are obtained that express similar markers to oocytes such as *Gdf9b*, VASA, and DAZL. To date the resulting OLCs remain unable to mature or be fertilized [12].

Although the OLCs remain unable to function the protocol used to form these OLCs may still have utility as an assay to study the formation and development of earlier stage germ cells within a closed *in vitro* system. The original publication discussing the formation of OLCs from somatic stem cells utilized fetal porcine skin as a stem cell source [8]. Expanding on that study the PGC-like cells formed early during induced differentiation were shown to be the precursors to the OLCs and express such PGC markers as OCT4, VASA, STELLA, C-KIT, and DAZL [13]. This data supports the potential of utilizing this system to study germ cell formation and differentiation *in vitro*. The protocol utilized to form OLCs from newborn mouse skin will be demonstrated in this video article. This protocol offers an *in vitro* model to study germ cell development that may provide a means to elucidate factors important for their proliferation and differentiation.

Protocol

1. Media Preparation

1. Prepare media 2-3 hr in advance of use and place with a loosened cap in the cell culture incubator where the experiment will be taking place. Media used in the present protocol:
    1. Prepare stem cell medium consisting of DMEM/F12 supplemented with 1 x B27, 40 ng/ml bFGF, and 20 ng/ml EGF.
    2. Prepare germ cell differentiation medium consisting of m199 supplemented with 0.05 IU FSH, 0.03 IU LH, 3 mg/ml BSA, 5 µl/ml ITS, 0.23 mM sodium pyruvate, 1 mg/ml Fetuin, and 1 ng/ml EGF. A base medium consisting of all additives but omitting FSH, LH and EGF can be prepared and stored at 4 °C for up to one week.

2. Stem Cell Culture Preparation

1. Isolate stem cells from newborn mouse pups as previously described[12]. Utilize stem cells at passage 2-4 for *in vitro* germ cell differentiation. The efficiency of OLC formation is directly related to the quality of the starting stem cells. It is best to start the differentiation as soon as the population appears clean and healthy which is generally at around passage 2. Further culturing the stem cells past passage 2 negatively affects the OLC formation efficiency (unpublished results).
2. 48 hr prior to initiating differentiation sub-culture the stem cells. Remove all the suspended spherical cell aggregates and spent media from the culture dish, using a serological pipette, and place in a 15 ml tube. It is important to only collect the suspended spherical aggregates of cells and not the cells attached to the bottom of the dish that are spontaneously differentiating.
3. Pellet the cells at 500 x g for 5 min., discard the supernatant, and resuspend in 500 µl of fresh stem cell media warmed to 37 °C. Gently pipette the aggregates to partially dissociate the cells. Do not aggressively pipette the cells as this will reduce cell viability. Wash the partially dissociated cells into 9.5 ml of fresh pre-warmed stem cell medium on a low attachment 10 cm cell culture dish.
4. Return the cells to a 37 °C, 5% CO_2 cell culture incubator for 48 hr prior to initiation of differentiation.

3. OLC Differentiation

1. Using a serological pipette remove the stem cells from the culture dish and place in a 15 ml tube, leave behind any attached cells. Pellet the cells at 500 x g and resuspend in 500 µl sterile PBS. Vigorous pipette the cells using a wide bore 1,000 µl tip into clumps not larger than 10-20 cells. Periodically remove a small amount of cells, during dissociation, and check under a white light microscope to confirm aggregated cells are in clumps of 10-20 individual cells. Over dissociation will result in reduced cell viability.
2. Add 9.5 ml PBS to the dissociated cells and add 15 µl of this cell suspension to a hemocytometer for cell counting. Pellet the cells at 500 x g for 5 min.
3. Re-suspend the cells at a concentration of $1.32X10^6$ cells/ml in differentiation medium.
4. Plate the cells by adding 500 µl per well into a flat bottom 24 well suspension dish.
5. Place the 24 well dish into a cell culture incubator at 37 °C in 5% CO_2 for 48 hr. Remove 250 µl medium from each well and place in a correspondingly labeled 1.5 ml tube. Add 250 µl fresh differentiation medium to each well. Centrifuge the spent medium at 500 x g for 5 min. and discard the supernatant. Resuspend any pelleted cells in 50 µl fresh differentiation medium and return to the corresponding culture well on the differentiation plate.
6. Every 48 hr change half the medium up to day 12 of differentiation.

Representative Results

Initially the cells attach to the culture dish bottom and spread out. At 42-72 hr into differentiation small suspended OCT4 positive cells form and proliferate (**Figure 1A**). Shortly after the visualization of these cells the majority will disappear and the attached cells will form dense colony aggregates on the culture bottom. Following a few days these aggregates will detach from the culture surface. In some of these aggregates a large cell can be observed (**Figure 1b** and **1c**). Upon initial visualization the cells will be ~35 µm in diameter. With continued culture the OLCs will grow to reach ~45 µm in diameter. These are the OLCs and can be collected with support cells or trypsinized to obtain OLCs without other cells attached (**Figure 1d**).

In order to confirm that the OLCs express similar transcripts as natural oocytes the OLCs can be collected and tested for the expression of such markers as *Zpc* , *Scp3* , *Cmos* , *Oct4* , *Bmp15*, and *Vasa*. While *Oct4* and *Vasa* are expressed in the undifferentiated stem cells several markers such as *Bmp15* and *Zpc* are oocyte specific. BMP15 is a member of the TGF-β family and is involved in oocyte and follicular development. The oocyte specific zona pellucida membrane structure is made up of several proteins including ZP3. The expression of these oocyte specific markers can be used to confirm the presence of OLCs in the differentiations. Furthermore, the expression of meiotic specific markers such as *Scp3* and *Cmos* can identify potentially meiotic cells within the culture system. In the example provided 10 OLCs and 10 oocytes were collected and direct cDNA synthesis performed (**Figure 2**). The resulting samples were then tested using semi-quantitative PCR. Comparing the expression of these markers to natural mouse oocytes we see differences in the levels of transcripts (**Figure 2**). Many markers expressed by oocytes will be expressed by OLCs if the differentiation was successful.

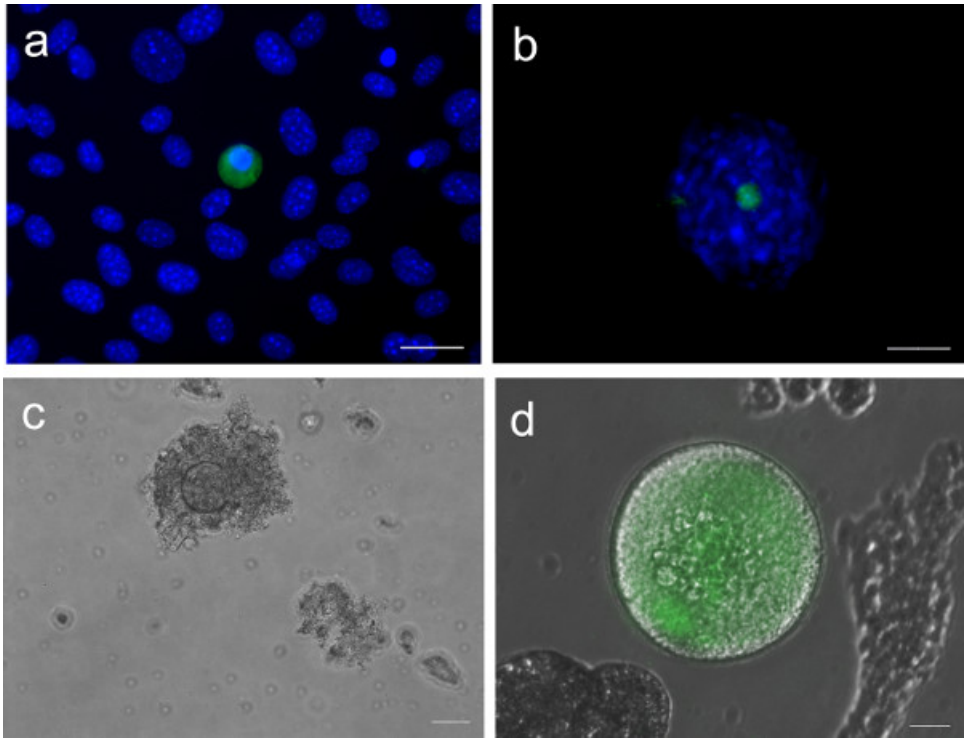

Figure 1. Common morphologies during induced differentiation of skin derived stem cells. a) During early stage differentiation cells positive for OCT4 (green) can be detected in the culture. Nucleuses are counter stained with Hoechst (blue). **b)** As differentiation progresses some cell aggregates will be seen with OCT4 (green) positive cells surrounded by OCT4 negative cells. Nuclear staining with Hoechst is also depicted (blue). **c)** A white light image of a follicle-like structure following detachment from the culture surface. **d)** During differentiation large OCT4 positive (green) oocyte-like cells can be seen either surrounded by cells or singly within the culture. Scale bars: a = 20 µm, b = 100 µm, c = 40 µm, d = 10 µm.

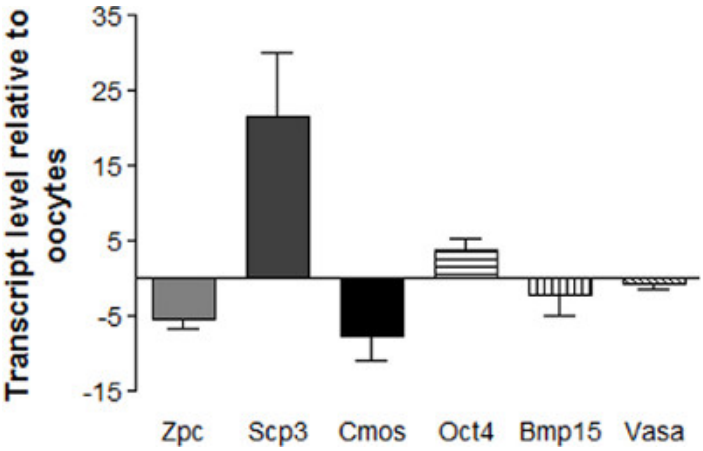

Figure 2. Transcript level in OLCs, compared to oocytes, of common oocyte markers. Representative real time PCR results showing the transcript level of common oocyte markers in OLCs. This comparison is resulting from 10 oocyte-like cells being compared to 10 newborn mouse oocytes.

Discussion

To date functional OLCs have not been developed using a completely *in vitro* assay. Recently a study by Hayashi *et al.* was able to produce offspring from PGC-like cells formed *in vitro* and transferred *in vivo* for further development[14]. Starting with ES cells or induced pluripotent cells they were able to form PGC like cells which, when recovered following *in vivo* transplantation were able to be matured, fertilized, and used to generate offspring [14]. This demonstrates that stem cells from various sources have the potential, under the correct conditions, to form functional oocytes.

Certain aspects of the culture system are critical to it functioning. The strain of mice utilized to isolate the stem cells is very important. This protocol was developed and used with B6 mice from the Jackson Lab (Stock #008214). These mice carry an Oct4-EGFP transgene allowing visualization of cells expressing Oct4 via the EGFP protein. When the protocol was attempted using stem cells from CD1 mice the efficiency was much lower and the OLC formation was less reliable. Currently the efficiency of OLC formation remains low even when using B6 mice. The stem cells utilized should be at passage 2-4 prior to initiating germ cell differentiation as the efficiency of germ cell formation is much lower when using later passage cells. The source of various reagents also has proven to be important.

The analysis of various mRNA transcripts in the OLCs may prove useful in optimizing the culture system. Using semi-quantitative PCR allows for the comparison of several markers expression levels between OLCs and oocytes (**Figure 2**). The results show that the OLCs express less *Zpc* than oocytes, which may explain the fragile nature and thinner zona pellucida membrane of OLCs. The expression of the meiosis marker *Scp3* is higher in the OLCs when compared to natural oocytes however *Cmos* is expressed at a lower level (**Figure 2**). This discrepancy in expression levels may partially explain the current failure of OLCs to complete meiosis. Other markers such as Bmp15 and Vasa are not significantly different between OLCs and oocytes.

The protocol described here provides a controlled *in vitro* method to study germ cell formation and differentiation. Currently the PGC-like cells generated are unable to form functional OLCs when maintained *in vitro*. When aggregated with newborn ovarian somatic cells and transplanted *in vivo* the PGC-like cells are able to form early stage secondary follicles. However when the OLCs are recovered they are still unable to be reliably matured and fertilized [12]. The utility in this assay comes from studying pre-meiotic germ cells derived from somatic stem cells.

Disclosures

No conflicts of interest declared.

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