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Retinoic acid promotes the proliferation of primordial germ cell–like cells differentiated from mouse skin-derived stem cells *in vitro*

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ABSTRACT

Skin-derived stem cells (SDSCs) have the potential to differentiate into gametes and are a potential resource for research and clinical applications. Sufficient amount of primordial germ cells (PGCs) is an important requirement for successful differentiation of SDSCs into gametes *in vitro*. Retinoic acid (RA), a vitamin A–derived small lipophilic molecule, promotes the growth of PGCs *in vivo*; however, the role of RA on the proliferation of PGC-like cells (PGCLCs) derived from SDSCs remains unknown. In this study, SDSCs were induced to differentiate into the embryoid body and cocultured with mouse fibroblasts to form PGCLCs. The proliferation of PGCLCs with the presence of various concentrations of RA was investigated *in vitro*. Immunofluorescence labeling showed that the 5-Bromo-2-deoxyUridine-positive ratio of PGCLCs was increased after the cells were treated with 5-μM RA, and flow cytometry results showed that the number of cells in the S phase was increased significantly. The messenger RNA expression levels of cell cycle–related genes, *CCND1* and *CDK2*, were also increased. Furthermore, RA effectively promoted the external proliferation of endogenous PGCs when 11.5-days postcoitum fetal mouse genital ridges were cultured *in vitro*. In conclusion, 5-μM RA promoted the proliferation of SDSCs-derived PGCLCs and endogenous PGCs. Our study will provide a valuable model system for studying the differentiation of stem cells into gametes *in vitro*.

1. Introduction

Owing to the tremendous societal burden and environment pollution in the world, many couples are faced with the puzzle of infertility [1]. Assisted reproductive technology is used for patients who cannot be treated with common cures, but for patients with azoospermia or abnormal eggs, assisted reproductive technology is not an option [1]. The differentiation of stem cells into germ cells brings a new prospect for treating infertility [2–8].

Many reports have suggested that stem cells have the potential to differentiate into germ cells under certain conditions [2–8]. The skin-derived stem cells (SDSCs) are abundant and are convenient to collect; therefore, the derivation of gametes from stem cells has become the new

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research hot spot [5,8–12]. Since 2006, *in vitro* differentiation of porcine [5,10,13–16], mouse [9,11,17], and even human [8] SDSCs into primordial germ cell–like cells (PGCLCs) and germ cell–like cells has been demonstrated. Primordial germ cells (PGCs), the progenitors of spermatogonia and oogonia, are the bridges between SDSCs and gametes. It is important to obtain sufficient PGCs to produce the foundational gametes *in vitro*. Linher et al. [14] have characterized PGCLCs generated from porcine SDSCs *in vitro*, and Shen et al. [15] have found that midkine is able to maintain a proliferative PGCLCs phenotype by suppressing the expression of meiosis-related genes in early germ cells. Furthermore, Park et al. [16] have reported that overexpression of DAZL in porcine SDSCs during the early stages of differentiation enhances the formation of PGCLCs, and Sun et al. [11] have found that mouse SDSCs derived from the skin of a newborn mouse have the potential to transdifferentiate into PGCLCs, which express key PGC markers and have similar dynamic epigenetic changes necessary for the development of PGCs *in vivo*. It is interesting that activin A can promote PGCLC formation from mouse SDSCs *in vitro* at early stages of differentiation [11].

Retinoic acid (RA) is a vitamin A (retinol)–derived, nonpeptidic, small lipophilic molecule that acts as a ligand for nuclear RA receptor (RAR). Some studies have suggested that RA has the ability to stimulate the expression of *Stra8* and meiosis in both sexes [18–20]. There has been extensive research on the mode of action of RA on meiotic initiation [21–23], but only a little attention from several groups has been given to the role of RA on PGC formation and proliferation [24,25]. Koshimizu et al. [24] have found that RA is a potent growth activator of mouse PGCs *in vitro*. Then, Morita and Tilly [25] found that RA acts as both a mitogen and a survival factor for germ cells during fetal mouse oogenesis *in vitro* and *in vivo*. It is interesting that the mouse embryonic stem cells can be induced into PGCLCs by RA and promote the self-renewal of PGCs *in vitro* [4,26–28]. Furthermore, Yu et al. have suggested that RA promotes the proliferation of chicken PGCs by increasing the expressions of cadherin and catenins or by activating the PI3K/Akt-mediated NF-kB signaling cascade [29,30]. However, the roles of RA on the growth and proliferation of PGCs differentiated from SDSCs have not been studied. The aim of the present study was to investigate the effect of RA on the proliferation of PGCs derived from mouse SDSCs *in vitro*.

2. Materials and methods

2.1. Animal care and mating

Mice used in this study were housed and bred under controlled lighting conditions (12L:12D). Housing females were mated with males overnight and then checked for vaginal plugs on the following morning. The chicken beta-actin promoter/enhanced green fluorescence proteins (CAG/EGFPs)–transgenic mice were used in this study [11,31]. All procedures involving animals were approved by the Institutional Animal Care and Use Committees of Qingdao Agricultural University.

2.2. Culturing of skin-derived stem cells

Skin-derived stem cells were obtained from the skin of newborn CD1-GFP mice [11,31]. The cells were cultured at 37 °C under 5% CO_2 in DMEM/F12 (HyClone, China) with 2% B27 (Gibco, USA), 20 ng/mL of EGF (Sigma, USA), 40 ng/mL of basic fibroblast growth factor (bFGF; Peprotech, Germany), and 1% penicillin–streptomycin combination (HyClone), and then, single cells were isolated after trypsinization. Media were changed every 4 days, and SDSC colonies were subcultured for two passages [11].

2.3. Differentiation of SDSCs into embryoid body cells

The skin-derived stem cell clones were isolated and were trypsinized into single-cell pellets at passage 2, and the single cells were cultured for additional 3 days in Medium 199 (Gibco) containing 3 mg/mL of BSA (Sigma), 1 mg/mL of fetuin (Merck, Germany), 5 μL/mL of insulin–transferrin–selenium (Gibco), 0.23-mM sodium pyruvate (HyClone), and 1 ng/mL of EGF (Sigma). Half of the media were changed every other day [11].

2.4. Culturing of mouse embryonic fibroblast and preparation of the feeder layer

The heads, limbs, tails, and viscera of the mice at 13.5 days postcoitum (dpc) were removed. The bodies were digested by trypsin to obtain single cells. The cells were washed with PBS and cultured in DMEM with high glucose (HyClone) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% sodium pyruvate (HyClone), 1% nonessential amino acids (Gibco), and 1% penicillin–streptomycin combination (HyClone). The fibroblasts of passages 2 and 3 were used as feeder cells. The cells were incubated for 2.5 to 3.5 hours at 37 °C in 4-mL mouse embryonic fibroblast medium containing 10 μg/mL mitomycin C (Solarbio, China). Then, the cells were washed with PBS for five times and digested by trypsin. Finally, mouse embryonic fibroblasts were cultured in a 24-well plate at a seeding density of 5 to 8 $\times$ 10^4 cells/well.

2.5. Differentiation of embryonic body cells into PGCLCs

Embryonic bodies (EBs) were digested into single cells and cocultured with feeder in DMEM with high glucose (HyClone) containing 10% FBS (Gibco), 0.23-mM sodium pyruvate (HyClone), 0.1-mM nonessential amino acids, 2-mM L-glutamine (Gibco), 0.1-mM β-mercaptoethanol (Sigma), 20 ng/mL of EGF (Sigma), 40 ng/mL of bFGF (Peprotech), and 40 ng/mL of stem cell factor (Sigma) at a seeding density of 10^5 cells/well. Primordial germ cell–like cells were treated with 0-, 2-, 5-, and 10-μM RA (Sigma) at Day 2.

2.6. Immunofluorescence

The differentiated cells were collected and fixed in 4% paraformaldehyde for 20 minutes. After washing three times in PBS, the cells were orderly permeabilized with PBS containing 0.5% Triton X-100 (PBST; Solarbio) for 10 minutes and blocked in PBST containing 10% normal goat