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Derivation of chicken primordial germ cells using an indirect Co-culture system

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ABSTRACT

Primordial germ cells (PGCs) are promising genetic resources for avian studies including modified animals. However, chicken PGCs are slow to proliferate and gradually lose germline competency after long-term culture, which hinders their application in avian biotechnology. Thus, we developed a robust method for the isolation and rapid propagation of PGCs using an indirect co-culture system. PGCs derived from a pair of embryonic chicken gonads were expanded to 1×10^6 within 2 weeks, and no sex bias was observed in. These PGCs presented high capacity of germline transmission and produced donor-derived offspring after injection into the chicken embryos. This system allows the efficient gene-banking of chicken species and can facilitate the production of chickens bearing a desired phenotype via genomic editing.

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1. Introduction

Chicken is an inexpensive meat and egg protein source and a resource for developmental biology and health science research [1,2]. Genomic modification can improve the productivity and development of disease-resistant chickens, the production of therapeutic proteins *in ovo*, as well as the establishment of animal models for the study of gene functions [3].

Primordial germ cells (PGCs) are gamete precursors that transfer genetic information from one generation to the next [4,5]. During early embryonic development, avian PGCs are guided by chemokine receptors and migrate to developing gonads through the vascular system [6], allowing isolation for expansion and genetic modification *in vitro*. Previous reports have revealed that germline chimeras are obtained by transplantation of PGCs isolated from germinal crescent, embryonic blood, or embryonic gonads [7–9], which may be promising tools for producing transgenic chickens or preserving genetic resources in endangered birds or domestic avian species [8]. However, procuring PGCs from embryonic tissues is inefficient, and the number of cells recovered is limited. Several culture systems have been developed to expand PGCs using conditional KnockOut (cKO) Dulbecco's Modified Eagle's Medium (DMEM) and co-culture with feeder layers, such as STO mouse fibroblasts, buffalo rat liver (BRL) cells [8,10] and chicken embryonic fibroblasts (CEF) [11]. Serum- and feeder-free cultures have also been developed by supplementing culture medium with ovalbumin [12]. However, chicken PGCs derived from these culture systems are slow to proliferate, and often freshly isolated PGCs cease to grow or are diminished even after a few days in culture. For cells that adapt to the culture system, it requires 4–8 weeks to establish a cell line ($>10^6$ magnitude) that stably proliferates [8,9,12,13]. Germline transmission of PGCs tends to decrease with extended culture duration [8,9], which decreases the efficient proliferation of chicken PGCs and hampers the implementation and use of biotechnological applications for genetic conservation.

In this study, we developed a robust method for the isolation and rapid propagation of PGCs derived from embryonic gonads. PGCs in more than 90% of the isolations grew to 1×10^6 in 2 weeks. Significant migration of PGCs to the embryonic gonads occurred after transplantation of the cells into chicken embryos, and donor-

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derived offspring were obtained, indicating the ability of PGCs to maintain germline transmission.

2. Materials and methods

2.1. Optimization of culture for the proliferation of chicken PGCs

Enhanced green fluorescent protein (EGFP)-PGC lines used for the optimization of culture proliferation (Fig. 1A and B) were previously obtained [14]. Briefly, these PGCs were derived from blood, cultured for 60 days, and EGFP modified using the piggyBAC transposon system. Then the EGFP-PGCs were cultured on mouse embryonic fibroblast (MEF) feeder cells using cKO culture medium in a saturated humidity atmosphere of 37 °C and 5% CO_2. Cells were in a good proliferative state on the day of transfer. PGCs were collected by gentle pipetting (without enzyme digestion), re-suspended with a different culture medium, and transferred into 96-well plates with or without MEF at a density of 1000 cells/well (for each culture system, n = 24). On days 1, 3, and 6 after seeding (Fig. 1A), proliferation of the EGFP-modified PGCs was assessed using the BioTek Cytation 5 Imaging Multi-Mode Reader (Thermo Fisher Scientific, Waltham, MA, USA) by counting the EGFP fluorescence points in each well (Fig. 1A). Population doubling time was determined using exponential regression curve fitting (www.doubling-time.com/compute.php). The composition of KO-DMEM(cKO), FACs, modified Knockout DMEM (mKO) medium used for optimization [12,14] is listed in Table 1.

2.2. PGC isolation and cell culture

All procedures involving animals were reviewed and approved by the Committee for Animal Care, Guangxi University (Nanning, China), and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Fertilized eggs were collected from Donglan chickens, a black feather chicken breed native to Guangxi Province, China. Embryonic gonads were isolated from 7-day-old chicken embryos (stage HH27-31), trypsinized with 0.05% trypsin-EDTA at 37 °C for 10 min, neutralized in DMEM/F12 supplemented with 10% fetal bovine serum FBS and plated in 24-well plates with 24-well transparent membrane (PET) inserts (1.0 μm; Millipore, Stafford, VA, USA). After 4–5 h incubation in 5% CO_2 at 37 °C, suspended cells were collected by gentle pipetting, and the DMEM/F12 was removed and added to different PGC culture systems (each embryo represented one sample). The PGC line derived was used for three different batches of fertilized eggs, and 5–10 embryos were used for each batch. The proliferation data from the three PGC lines used in the different culture systems were collected to construct the growth curves (Fig. 3A).

2.3. Cryopreservation of modified knockout-freshly isolated cultured PGCs

Modified knockout-freshly isolated (mKO-FI) PGCs were cryopreserved in full culture medium (mKO) supplemented with 10% FBS and 10% DMSO, with 0.5×10^6 cells in each vial. The cell recovery curve in Fig. 4A were constructed from three PGC lines (n = 3). The three cell lines went through a freeze-thaw cycle, were cultured for another 10 days, and then were re-injected into recipient chicken embryos to verify germline transmission ability.

2.4. RNA and DNA extraction and PCR

Total RNA was extracted from PGCs and CEF (used as a control in reverse transcription (RT-PCR)) using the Omega RNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA). The first-strand cDNA was synthesized using a Reverse Transcription Reagent Kit and gDNA Eraser (Takara Biomedical Technology, Beijing, China), which removed contaminating genomic DNA. Genomic DNA was prepared using a DNA Extraction Kit (Tiangen Biotech, Beijing, China) from the blood and tissues of chicken. The genomic DNA from male and female CEF cell lines were used as controls for PCR-sexing. PrimeSTAR Max DNA Polymerase (Takara Biomedical Technology) was used for PCR.

2.5. Immunocytochemistry

Freshly isolated PGCs were collected from culture by gentle pipetting, followed by centrifugation and fixing in 4% paraformaldehyde (Sigma, St. Louis, MO, USA). Freeze-thawed PGCs were collected after 7 days of recovery, and then fixed in 4% paraformaldehyde. Primary antibodies for stage-specific embryonic antigen 1 (SSEA-1; Hybridoma Bank, University of Iowa, Iowa City, IA) and Deleted in Azoospermia-Like (DAZL; prepared in our laboratory) were diluted in blocking solution (1:20 phosphate-buffered saline, 1:1000 5% FBS). PGCs were incubated in primary

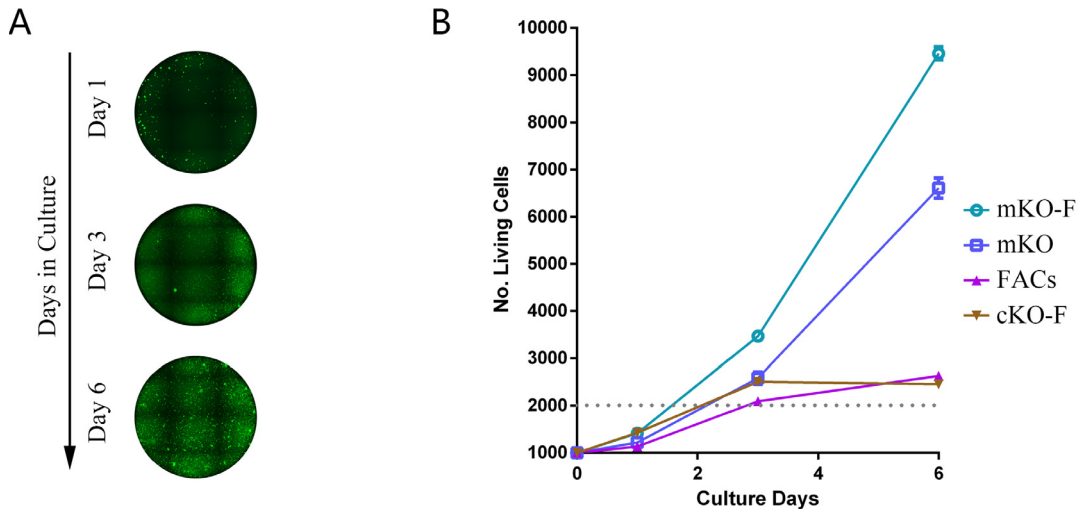

Fig. 1. Proliferation of GFP-modified PGCs in different cultures. A. GFP-modified PGC proliferation was imaged and counted using a multi-mode plate reader. **B**. Proliferation of GFP-PGCs in cKO-F, FACs, mKO, and mKO-F cultures (n = 24).

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