Animal Reproduction Science 171 (2016) 36–40

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Cholesterol induces proliferation of chicken primordial germ cells

Dongyang Chen, Meijuan Chen, Zhenping Lu, Mengmeng Yang, Long Xie, Wenxin Zhang, Huiyan Xu, Kehuan Lu, Yangqing Lu *

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi High Education Laboratory for Animal Reproduction and Biotechnology, Guangxi University, Nanning, Guangxi, China

ARTICLE INFO

Article history:
Received 30 March 2016
Received in revised form 18 May 2016
Accepted 20 May 2016
Available online 22 May 2016

Keywords:
Chicken PGCs
Proliferation
Cholesterol
Sonic hedgehog signaling

ABSTRACT

Primordial germ cells (PGCs) are the precursors of sperm and eggs and may serve as suitable cells for use in research in developmental biology and transgenic animals. However, the long-term propagation of PGCs in vitro has so far been plagued by the loss of their germ cell characteristics. This is largely because of the scarcity of knowledge concerning cell division and proliferation in these cells and the poor optimization of the culture medium. The sonic hedgehog (SHH) signaling pathway is involved in proliferation of many types of cells, but little is known about its role in chicken PGCs. The results of the current study indicate that the proliferation of chicken PGCs increases significantly when cholesterol, a molecule that facilitates the trafficking of HH ligands, is supplemented in the culture medium. This effect was attenuated when an SHH antagonist, cyclopamine was added, suggesting the involvement of SHH signaling in this process. The characterization of PGCs treated with cholesterol has shown that these cells express germ-cell-related markers and retain their capability to colonize the embryonic gonad after re-introduction to vasculature of stage-15 HH embryos, indicating that proliferation of PGCs induced by cholesterol does not alter the germ cell characteristics of these cells.

1. Introduction

Primordial germ cells (PGCs) are the precursors of sperm and eggs. They serve as the vehicle that passes genetic information from one generation to the next (Petitte et al., 2004; van de Lavoir et al., 2006). PGCs are specified from somatic cells in early embryonic stage and determine germ cell fate (Ohinata et al., 2009; Hayashi et al., 2011, 2012; Irie et al., 2015). Because these cells are totipotent, they could be genetically modified and give rise to fully transgenic progenies (Park and Han, 2012; van de Lavoir et al., 2012; Schusser et al., 2013). However, due to the small number of such cells in early-stage embryos, and their intendancy to undergo proliferation in vitro, long-term culture of chicken PGCs without loss of germline potential remains challenging. There is a plethora of signaling pathways involved in cell proliferation, but little has been elucidated in avian PGCs. The system of culturing chicken PGCs is far from ideal for proliferative culture, especially when these cells are freshly isolated or under a clonal expansion condition for genetic screening. This dilemma has hindered the use of PGCs in the study of developmental biology and transgenic animal research.

The hedgehog (HH) signaling pathway plays an important role in the development of vertebrates (Zuniga et al., 1999) and invertebrates (Besse et al., 2005; Hartman et al.,

* Corresponding author.
E-mail address: luyangqing@126.com (Y. Lu).

http://dx.doi.org/10.1016/j.anireprosci.2016.05.011
0378-4320/

2013), including cell proliferation, differentiation, and the formation of the organization (Ingham and McMahon, 2001). Results have shown that HH signaling is involved in regulation of germ-line proliferation in the ovaries of Drosophila larvae and *Caenorhabditis elegans* (Shim et al., 2002; Sahai-Hernandez and Nystul, 2013). Cholesterol, an important structural molecule of cellular membrane, is not only involved in the process and spreading of signaling domain HH-N, but it is also required for long-range signaling activity. The HH signaling pathway has been found to be conserved in chicken and to play a significant role in chicken development (Fukuda et al., 2003; Bardet et al., 2010). Interestingly, cholesterol is enriched in the egg-yolk, the natural niche of chicken PGCs. The role of cholesterol in chicken PGC proliferation, and the specific pathways in which it is involved, are unknown. The purpose of this study was to investigate the effect of cholesterol on cell proliferation and its related signaling pathway in chicken PGCs.

2. Materials and methods

2.1. Isolation and culture of PGCs

All procedures for use of animals described here were reviewed and approved by the Guangxi University Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. Fertilized eggs from Guangxi Yellow-feather chicken (Gallus gallus) were collected from Guangxi University experiment farm and incubated in an egg incubator at 37.8 °C and 60% humidity for 55 h. A 1 cm-diameter window was made on the blunt end to expose the embryos. Microglass needle is used to collect the circulating blood and transferred to a 6-well plated preseeded with irradiated Buffalo Rat Liver (BRL) feeder. PGCs are cultured in KO-DMEM (Invitrogen) containing 4 ng/mL bFGF, 7.5% defined FBS (Hyclone), 2.5% chicken serum (Sigma), 1 × pen/strep (Gibco), 1 × GlutaMAX (Gibco), 1 × GS nucleoside supplement (Millipore), and 0.1 mM β-mercaptoethanol (Sigma). The medium was changed every other day, and cells were subcultured every 4–5 days upon confluence.

2.2. Flow cytometry analysis

A total of 1×10^6 PGCs were collected and fixed in 70% ethanol for 30 min. Fixed cells were treated with 100 μg/ml RNase A (Tiangen) and 50 μg/ml propidium iodide (Sigma) at 37 °C for 30 min in a dark room. Then the stained cells were analyzed for the distribution of cell cycles by flow cytometry (BD FACS CELLULAR).

2.3. RNA isolation and RT-PCR

RNA was isolated using Total RNA Kit I (Omega) per the manufacturer's instructions. cDNA was synthesized using a FastQuant RT Kit (TianGen). Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) were performed using primers listed in Supplementary Table S1.

2.4. Immunocytochemistry

Cells were fixed in 4% PFA for 15 min and smeared on a glass slide marked with hydrophobic barrier. The smeared cells were blocked in 6% horse serum for 45 min, followed by incubation with primary and then secondary antibodies prepared in a blocking solution at room temperature for 60 min, respectively. The primary antibodies used in this study were SSEA1 (Developmental Studies Hybridoma Bank, MC-480). Secondary antibodies were goat anti-mouse IgM conjugated with AlexaFluor 594 from Invitrogen (A21044).

2.5. Cell injection and migration

Chicken embryos incubated for 55 h were used as host for PGC injection and migration assay. A 1 cm-diameter window was made on the blunt end above the air to expose the embryos. About 1×10^4 cells were injected into the bloodstream of embryos using a microglass needle. Eggshells were sealed with parafilm, and embryos were transferred to an incubator. After incubation for 5 days, embryos were cut open and the gonads were isolated and examined for cell migration under a fluorescence microscope (Olympus DP73).

2.6. Statistical analysis

Three biological replicates were performed in cell proliferation and inhibition experiments, each with three technical replications. All data are expressed as mean ± S.D., and a 2-way ANOVA test (GraphPad Prism 5.0) was used for analysis of significance. $P < 0.05$ was considered a significant difference.

3. Results

3.1. Cyclopamine inhibited the proliferation of chicken PGCs

To confirm that the SHH pathway is conserved in chicken and plays a role in proliferation of chicken PGC, cyclopamine (CyA), an antagonist of SHH signaling was added to the culture of PGCs and incubated for 5 days. Results showed cell counts to be similar in groups treated with CyA at 1.0 μM, 5.0 μM, and 10 μM, but all significantly lower than control group ($P < 0.05$), demonstrating that PGC proliferation was significantly inhibited by CyA (Fig. 1).

3.2. Cholesterol induced PGCs proliferation, and cyclopamine diminished it

Cholesterol was added to the culture of PGCs at 0.5 μM, 1.0 μM, and 2.0 μM and incubated for 5 days. Cell counting showed there to be a gradual increase in cell number along with the increase in cholesterol concentration. The cell count in group treated with 2 μM cholesterol was significantly higher than in control ($P < 0.05$) (Fig. 2A). Cell cycle distribution analysis showed accumulation of cells in the S and G2/M phase and a decrease in the proportion of cells in the G1 phase after incubation with cholesterol at 2.0 μM for 5 days (Fig. 2B).